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Germline development in *Platynereis dumerilii* and its connection to embryonic patterning

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To my mother and my brother for their encouragement and example

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1 INTRODUCTION

1.1 The evolution and phylogeny of Metazoa

“Nothing makes sense, except in the light of evolution" (Dobzhansky, 1973). This famous quote is certainly an exaggeration, but in many cases evolution is indeed a very useful concept to solve the puzzles of biology. Perhaps no other field of biology profits from this explanatory power of evolution as much as the field of developmental biology. Since Haeckel has proposed the fundamental connection between ontogeny and phylogeny (1880), developmental biologists have discovered that many seemingly complicated developmental processes – such as formation of the germ cells- are easier to explain when one takes their evolution into account. Although the tools of biology have changed over the last 200 years, this methodological principle continues to be useful in our times. While previous developmental biologists have drawn conclusions on ontogeny by observing and mainly comparing morphological traits. We can nowadays use large amounts of molecular information to address more complex questions that were not possible to answer by only evaluating morphological data.

In order to address the evolutionary aspects of developmental problems, we have to compare our developmental studies to a broad variety of animal model systems. Only if we study how embryos of related species develop, we can distinguish the important conserved aspects of their development. Evolution never or very rarely gives rise to a completely new or novel structure. New structures rather arise through a line of small modifications of an existing structure. Therefore, comparing the data between differently related organisms can also help us to unravel the small steps that lead to the very different morphologies we observe today. In order to translate these differences into a consecutive series of small steps, one prerequisite is that we know about the phylogenetic relationships of the species we investigate, and these are summarized in Figure 1.

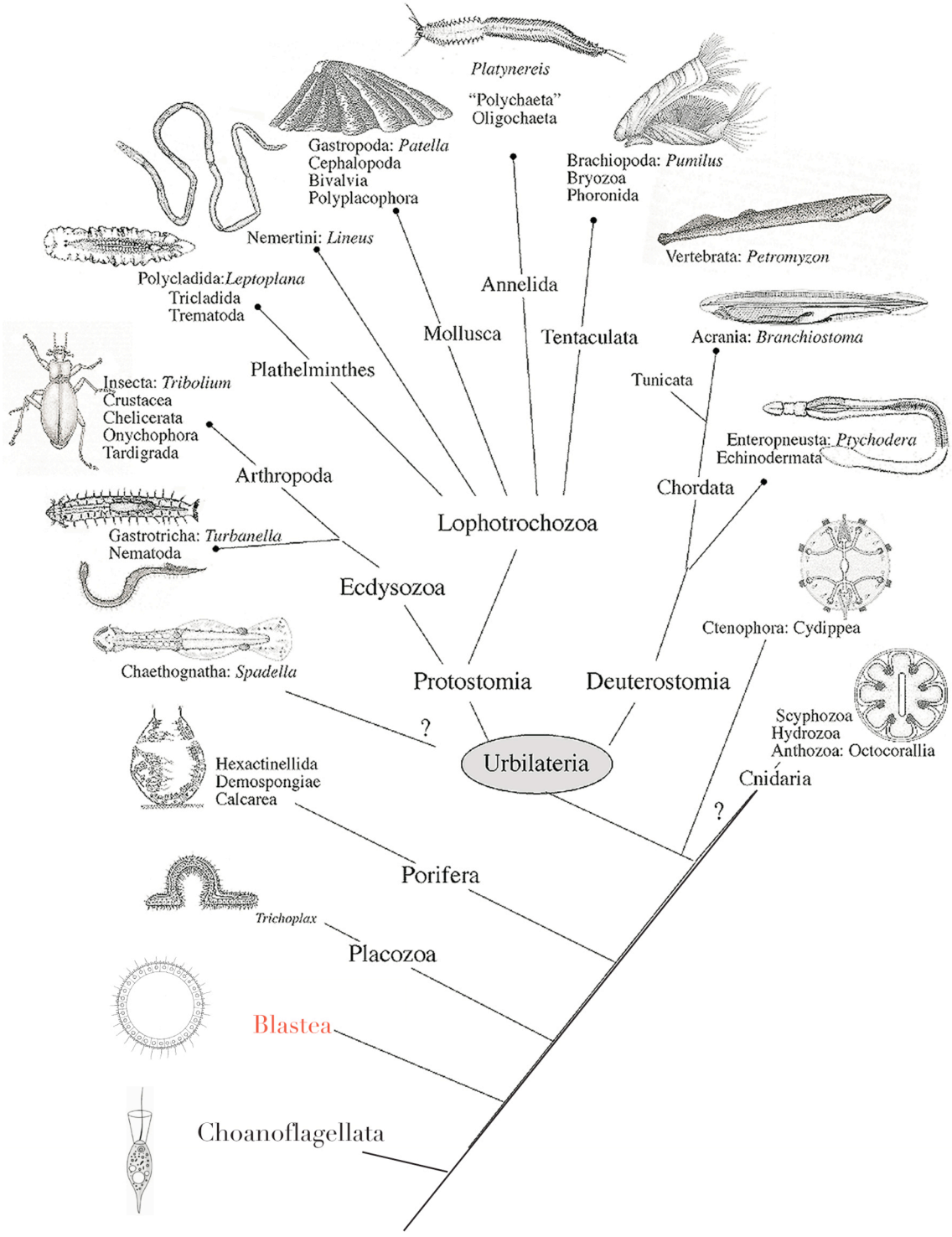


Figure 1. Simplified phylogenetic tree of Metazoa

The tree shows the position several organisms in a simplified phylogenetic tree based on (Aguinaldo et al., 1997; Halanych et al., 1995; Nielsen, 1995; Willmer, 1990). The tree has been modified after Arendt and Wittbrodt, 2001, including the Blastea (in red), based on the Blastea Theory of Ernst Haeckel. *Platynereis dumerilii* belongs to the lophotrochozoans (top of the tree), a branch of the Protostomia.

1.2 Mechanisms for the establishment of the germ cells in Bilateria

Organisms that reproduce sexually use specialized cells, the germ cells, for reproduction. These cells derive from a small group of germline progenitors called the primordial germ cells (PGCs) which are set aside from other cell lineages in early embryogenesis (Wylie, 1999). During gastrulation, these germline progenitors move inside the embryo, usually in association with the developing gut, and eventually migrate out of the gut into the somatic gonad. These PGCs then undergo extensive proliferation before differentiating into gametes.

Two broad strategies exist in metazoans to specify the germ cell lineage. The first strategy is the asymmetric **inheritance of maternal factors** that comprise the germ plasm, which contains particles called the germ granules. This strategy is used to specify PGCs in many organisms such as nematodes, insects, crustaceans and Anuran amphibians (Nieuwkoop and Sutasurya, 1979; Nieuwkoop and Sutasurya, 1981).

Many of the factors necessary for germ cell development reside in the germ granules (Eddy, 1975). Germ plasm is morphologically similar in all organisms that contain it, and consists of electron-dense germinal granules, mitochondria and ribosomes (Czolowska, 1972). By definition, **germinal granules** are cytoplasmic, non-membranous RNA/protein complexes aggregated in the primordial germ cells of many higher eukaryotes (Eddy, 1975). In the following, two examples are given of organisms in which the primordial germ cells are established by the localization of such germ granules.

In the nematode, *C. elegans*, the germinal granules are called P granules (Strome and Wood, 1982; Strome and Wood, 1983; Wolf et al., 1983). P granules are dispersed throughout the cytoplasm in the mature oocyte, segregate to the posterior cortex after fertilization and continue to segregate into the P germline lineage in the early embryo until they surround the nucleus of the P4 germ cell progenitor at the 16- to 24- cell stage. These granules remain in the germline of the worm throughout all its life cycle, surrounding each germ cell nucleus during mitotic proliferation and gametogenesis.

In ***Drosophila***, the germinal granules are called polar granules, and transplantation of cytoplasm containing these granules can induce germ cells ectopically, for example at the anterior position of the fly embryo (Illmensee and Mahowald, 1976). Mutations that affect

the polar granule assembly and germ cell formation include genes of the "posterior group": *vasa*, *tudor*, *staufen*, *valois*, *mago-nashi*, *oskar* and *nanos* (Johnston and Nusslein-Volhard, 1992). *Vasa* and *nanos* are two genes that will be analysed in this work. Both genes are expressed during oogenesis; and their function involves a cascade of several transcription factors, among other proteins. Since the interrelationships of these factors will be important in the later context of this study, they are summarized in the following section.

During early *Drosophila* germline determination, the TGFα signalling molecule gurken establishes polarity within the oocyte along both the antero-posterior and dorso-ventral axes (reviewed in Nilson and Schupbach, 1999). Subsequently, at the posterior end of the oocyte and early embryo, a specialized region of cytoplasm, called pole plasm, accumulates RNAs and proteins required for germline determination. Their assembly is initiated through the localized translation of *oskar* mRNA (Ephrussi et al., 1991; Kim-Ha et al., 1991). Vasa protein and *nanos* mRNA are among the molecules that accumulate in the pole plasm down-stream of oskar protein. Localized translation of *nanos* mRNA in the pole plasm produces a nanos protein gradient that is essential to determine abdominal fate. Nanos links germ cell development to posterior somatic patterning of the embryo (Lehmann and Nusslein-Volhard, 1987; Wang and Lehmann, 1991). *Oskar*, *gurken* and *nanos* RNAs are all under complex translational regulation in the developing oocyte, mediated through cis-acting elements in their UTRs. Bruno is involved in repressing translation of *oskar* and *gurken* (Filardo and Ephrussi, 2003; Nakamura et al., 2004). Vasa could play a role in *gurken* translational control (Ghabrial and Schupbach, 1999). Generation of nanos protein in the posterior of the embryo is essential to allow abdominal segmentation, whereas preventing nanos accumulation in the anterior is essential for normal head and thoracic segmentation. Smaug acts as a translational repressor by binding *nanos* mRNA at a stem loop structure located within its translational control element. This activation in the pole plasm is genetically dependent on vasa and oskar (Dahanukar and Wharton, 1996), two of the 'posterior group' gene products known to be required for assembly of the pole plasm.

In contrast to these well-studied examples of asymmetric inheritance of maternal factors, a structure such as the germ plasm has not yet been observed in all organisms. Instead, in these organisms, a second mechanism of germ cell determination is thought to specify the germ lineage during embryonic development, namely **extrinsic signalling**. One molecular

mediator of such extrinsic signalling is the Bone Morphogenetic Protein (BMP) family, which is implicated in formation and maintenance of primordial germ cells in mice (Lawson et al., 1999; Ying et al., 2001).

In mice, primordial germ cells are generated in the epiblast. They consist of putative pluripotent cells that have been identified as a small cluster of alkaline phosphatase-positive cells in the extra-embryonic mesoderm at 7.25 days post coitum (dpc). These cells then move down to the embryonic mesoderm at the posterior end of the primitive streak at 8.0 dpc, then they migrate through the hindgut endoderm and colonize the developing genital ridges at 10.5-11.5 dpc. Here they are destined to form germ cells (Eddy and Hahnel, 1983; Ginsburg et al., 1990). The determination of the germ cells in mice is believed to be independent of maternal factors that would be localized in a specific region of the cytoplasm. In contrast, it is reasonable to believe that in some organisms fate determination of germ cells -such as sex-determination- is rather dependent on cell-cell interactions with gonadal somatic cells (reviewed by McLaren, 2003).

Since both types of mechanisms, inheritance of maternal factors and extrinsic signalling, occur in different phyla across the animal tree, it is difficult to decide which of them- if any- may be the more ancestral type of germ cell specification. Along with the prominent position of *Platynereis* as an ancestral-type of organism (see below), this was a strong motivation to investigate the mode of germ cell specification in this animal.

1.3 Connection between the specification germ cells and the establishment of embryonic axis

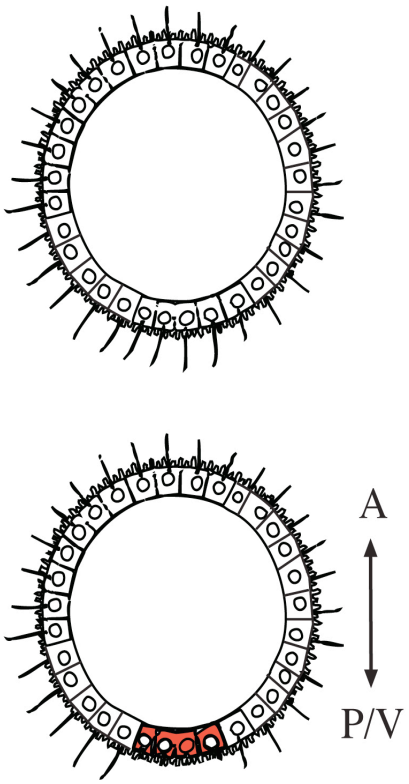
Independent of the mechanisms by which they get specified, germ cells are essential in almost all animal groups. But how did they arise initially at the dawn of Metazoa? A division of labour among cells can only occur after the occurrence of multicellularity. In multicellular organisms some cells can specialize. Ernst Haeckel in his Colonial or Blastea Theory first proposed this in 1874. Metschnikoff later modified this theory in 1883 and then by Libby Hyman in 1940. The theory proposes that colonial flagellated protozoans, probably similar to *Proterospongia*, were the ancestors of modern metazoans. These simple spherical organisms enclosed a cavity filled with fluid that served for exchanging nutrients (Figure 2A). The colony comprised a hollow sphere of flagellated cells that developed anterior and posterior locomotor orientation, along with the specialization of

some cells that would give rise to the next generation, the germ cells. These cells were distinct from the rest of the cells, which became specialized somatic cells that were responsible for collecting nutrients. Since the first cells specialized in reproduction were likely to occur on one pole only, this labour division correlated with the establishment of the first body axis (Figure 2B). Haeckel referred to this protometazoan as "Blastea" (see its hypothetical phylogenetic position in Figure 1- red).

The "blastea" or "colonial" theory is plausible since we find several extant living colonial protozoans that exhibit these characteristics (e. g., choanoflagellate protozoans) and the body wall of many lower metazoa such as poriferans and cnidarians, bear flagellated (or mono-ciliated) cells. A paradigm of the blastea is the alga *Volvox carteri*, which was thought by Haeckel to be a relic of the blastea stage of evolution (Figure 2C). *Volvox carteri* usually contains 2000 small, biflagellate somatic cells in the surface, and 16 large internally located asexual reproductive cells, called gonidia. These two cell types exhibit an exact germ-soma division of labour, since the flagellated somatic cells provide motility and collect nutrients, whereas the gonidia never develop flagella, but are responsible for reproduction (reviewed by Kirk, 1997).

According to Haeckel's theory, the Blastea then probably underwent invagination giving rise to the Gastrea, which is a double-layered gastrula-like organism (Nielsen, 2001).

Following the reasoning of Haeckel's biogenetic law – that ontogeny recapitulates phylogeny-, the gastrula of many modern metazoa is a recapitulation of this original gastrea form. In addition, diploblastic animals like cnidarians (Figure 1) can be interpreted as gastrea-type organisms that – in contrast to the bilaterian gastrula – neither acquire a "true" third germ layer nor develop "apparent" bilateral symmetry (Finnerty et al., 2004; Spring et al., 2002). Another line led to the Bilateria with the hypothetical Urbilateria as common ancestor of deuterostomes and protostomes. These animals are always triploblastic (Figure 1).



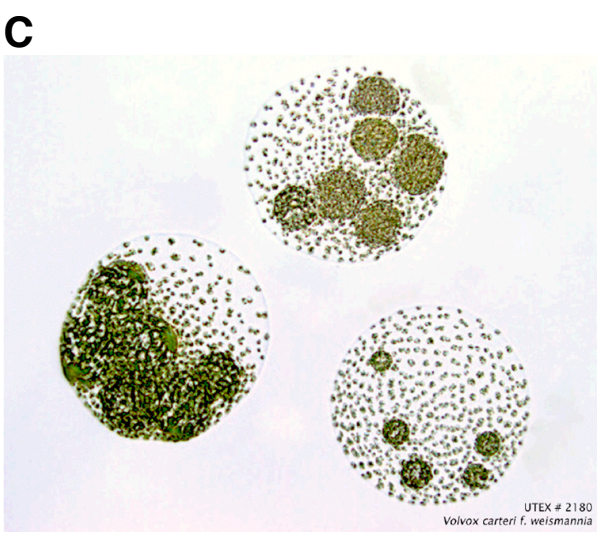


Figure 2. Representation of Haeckel's Blastea and the first axes formation in Metazoan Evolution

(A) In the hypothetical basic multicellular organism, all cells were equal. (B) Division of labour results in two different cell types, the somatic cells (responsible for collecting nutrients) and the germ cells (giving rise to the next generation) (C) *Volvox carteri* - a colonial flagellate. In this case, cells destined to become gonidia are set-aside early in development and never have somatic characteristics. The smaller somatic cells resemble *Chlamydomonas*. **A** stands for **A**nterior or **A**nimal pole, and **P/V** stands for **P**osterior or **V**egetal pole. Picture taken from http://www.bio.utexas.edu/research/utex

Indeed, molecular evidence argues that the mechanism used to establish the germ cells in the ancestor of the metazoa is the one also used to define the first body axis. The players in each species might vary to a certain degree. However, there are molecular conserved factors in all the three groups of the Bilateria. In *Drosophila* oocyte, the determinants - including *nanos* mRNA and vasa protein - that specify the germ cells are located in the posterior pole. Mutations on these determinants affect the formation of the posterior segments (Wolpert, 2001). The *Xenopus* egg stores factors involved in specifying the germ cells also in one region, in the vegetal hemisphere (Wolpert, 2001).

1.4 Platynereis dumerilii, an emerging model organism

1.4.1 Suitability of Platynereis as a model for cross-bilaterian comparisons

Developmental molecular studies of species belonging to the group of the lophotrochozoans are rare, understanding the need for a model system for this group of

animals. From its phylogenetic position, such a model is expected to greatly facilitate comparative evolutionary studies. Ideally, such a model should combine practical advantages – like breed ability in the laboratory-, with ancestral-type features. The polychaete *Platynereis dumerilii* meets these requirements. Not only can it be maintained in the lab, it also displays many ancestral features.

Among these ancestral-type features are the following:

- It develops indirectly through a primary ciliated larva- called trochophora in the spiralians-, which is not just ancestral for its group but also for the hypothetical Urbilateria (Arendt et al., 2001).
- Homonomous segmentation (Patel, 2003; Prud'homme et al., 2003; Stollewerk et al., 2003).
- Lifelong segment proliferation.
- Spiral cleavage.
- Its central nervous system (CNS) exhibits the classical rope-ladder-like organization, which is considered to be of ancestral type.
- Its blastopore lip forms both the anus and mouth, which is considered as prototype of protostomes (Arendt and Nübler-Jung, 1997).
- It has a prototypic closed circulatory system with a pulsatile dorsal vessel ("heart"), as most of the annelids have.

In addition to this, fossil records dated from the early Cambrium (Conway Morris, 2000) show organisms with features similar to that of *Platynereis*, consistent with the idea that Platynereis could be something like a "living fossil".

Furthermore, the following features make *Platynereis* a suitable organisms for molecular developmental studies:

- It has been successfully bred in the laboratory for over 30 years.
- It has a moderate life cycle of 3 to 6 months, which is well in the range of common vertebrate model systems (e. g., zebrafish – 3 to 6 months).
- It provides several hundreds of offspring per fertilization.

- Its cell lineages and development have been well described (Ackermann, 2002; Dorresteijn et al., 1993).

In addition to this, the fact that *Platynereis* has not changed its original habitat –the sea – suggests that not just its morphology still resembles the one of its ancestors but also its physiology and perhaps its genome. Its genome is relatively small of 900 to 1000 Mb (Gambi et al., 1997; Gregory, 2001) with a tendency of one single gene copy. In addition to this, the sequence of the genes seem to have evolved at a steady rate - represented in the phylogenetic trees by the short branch lengths –when compared to rapid diverging species, such as *Drosophila* and *C. elegans* (Tessmar-Raible, 2004).

Consistent with the suitability of *Platynereis* for comparative analysis, many efforts exist to increase sources of information and molecular resources for further studies in *Platynereis*. A wholemount in situ screening is being carried out by a consortium of five developmental biology laboratories in Europe on different embryonic developmental stages (24, 48 and 72hpf). This reports the expression pattern of several genes and an EST sequencing project will cover the sequence analysis of these genes (this information will be soon available as *Platynereis* expression pattern database, PEPD). In addition to this, Génoscope – the French National Sequencing Agency- has accepted to start sequencing the *Platynereis* genome within the next year. Several attempts to achieve loss- and gain of functional studies were performed and comprise: single cell injections, blastomere laser ablations and parental RNA interference.

Hence, *Platynereis* is one of the best possible choices for a comparative model system, ideally positioned to address evolutionary questions that would not be possible to answer by taking into consideration only data from the ecdysozoans (like *Drosophila* and *C. elegans*) and deuterostomes (like *Ciona*, zebrafish and mouse).

1.4.2 *Life cycle and embryonic development of Platynereis dumerilii*

Platynereis dumerilii is a member of the polychaetes, an ancient subgroup of the Annelida. Polychaete stands for poly=many and chaeta=bristles. It is a worm with many bristles in its limbs and lives in shallow waters along the seacoast. Its way of development is canonical for the polychaetes (shown in Figure 3, 8-cell stage). Gametocytes float freely in the coelomic cavity of the mature worm. During oogenesis the oocytes grow reaching a diameter of 160μm and arrest in prophase I of meiosis. The sperm shape belongs to the

primitive type, with a round head and tapered acrosome. Fertilization takes place in open water during the period of new moon. Females spawn by releasing several hundreds of oocytes through their body wall while males release the sperm trough the anal rosettes. Unequal spiral cleavage starts after 110-120mpf (minutes post fertilization) and epibolic gastrulation starts with the proliferation of the two blastomeres, so called somatoblasts - 2d and 4d. The 4d blastomere proliferates forming the mesodermal bands, which give rise to the mesodermal tissue. In contrast, the 2d blastomere is the precursor of the ventral plate, ectoderm and chaetal sacs (Ackermann, 2002). The ventral plate is the future neurogenic region. Rapid proliferation of the 2d lineage gives rise to a posterior growing area of numerous ectodermal cells that form the ventral plate (Wilson, 1892) from which the ventral cord will be formed. Additionally, the lateral 2d descendants invaginate and form the chaetal sacs. The ventral plate extends enclosing the embryo, overgrowing the four yolky endoderm precursor cells. At the same time, the mesodermal bands extend inside the embryo. This process is called epibolic gastrulation. The outcome of gastrulation is the trochophora larva (Dorresteijn, 1990). The trochophora undergoes metamorphosis, giving rise to a three segmented young worm that exhibits most of the body parts present in an adult worm (=juvenile), such as the stomodaeum –the precursor of the adult mouth, and the growth zone – from which the new segments will bud. After 3 to 6 months of growth, the adult worm enters epitoky, restructuring its parapodia to a shape more suitable for swimming. This facilitates swimming of the mature worm to the water surface where the gametes are shed (Hauenschild and Fischer, 1969). Females are recognized by their yellow colour while males are rather pink. The length of an adult or a mature worm is 3-6 cm.

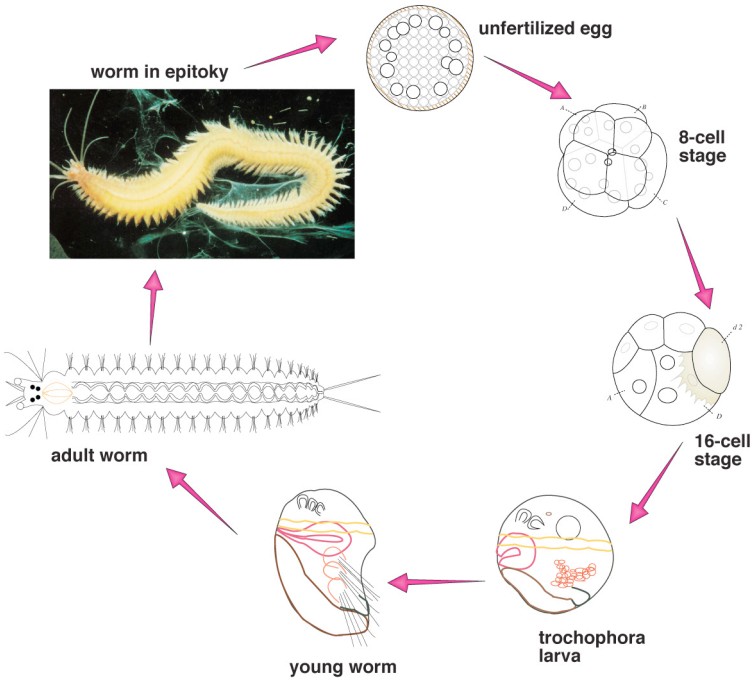


Figure 3. Life cycle of *Platynereis dumerilii*

The egg undergoes unequal spiral cleavage after its fusion with the sperm. This cleavage is represented by the 8- and 16-cell stage. At 16-cell stage the precursor of the ventral plate buds from the D blastomere. During gastrulation the trochophora larva forms. It then metamorphoses, giving rise to the young worm, which displays most of the body parts of an adult worm. The new segments of the young worm bud from the growth zone. Maturation starts when worms enter epitoky, in which stage the worm changes its colour and the shape of the parapodia, and softens its cuticle. Males have a pink colour, while females are yellowish, as shown in the picture. Photo taken by A. Dorresteijn.

To facilitate the identification of the main body regions of *Platynereis* larva, a schematic representation of the stages 15hpf and 48hpf is illustrated in Figure 4, along with a brief description of the most noticeable features.

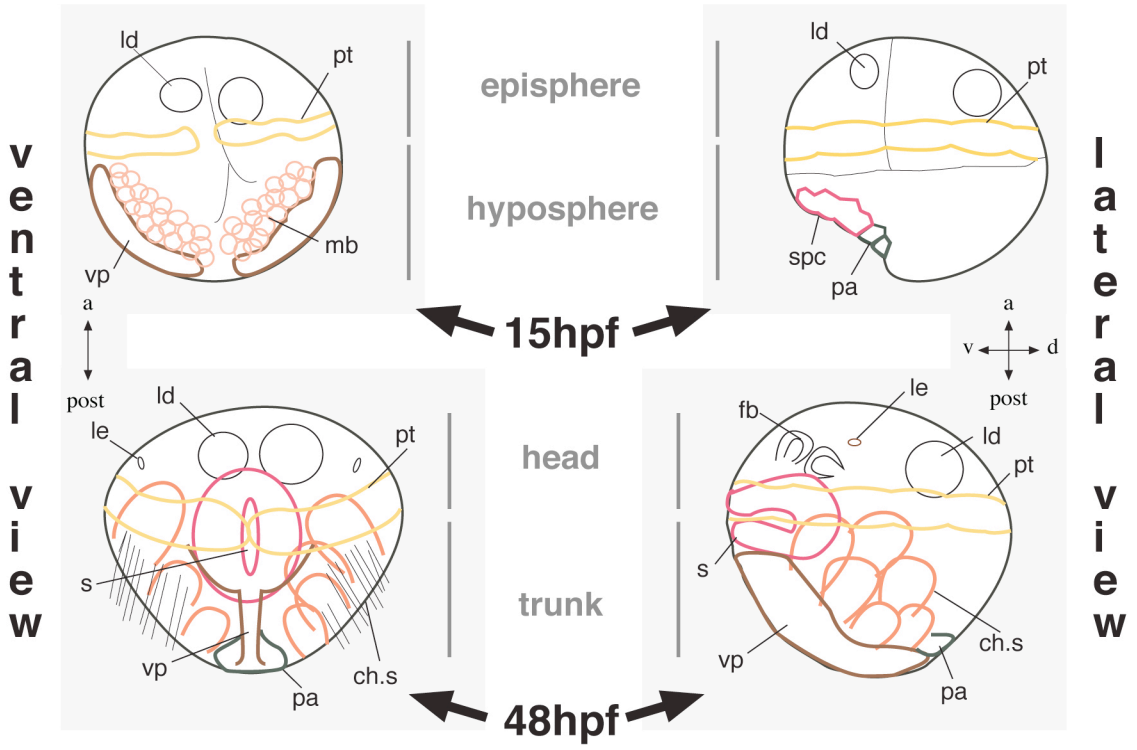


Figure 4. Introduction to the morphology of the *Platynereis dumerilii* larva and its most common views

At **15hpf** –the prelarval stage- the embryo is divided into the upper region (episphere), that contains 4 lipid droplets, the developing brain (including the larval eye region) and the ciliary bands (prototroch), and lower area (hyposphere), where we find the ventral plate, stomodaeal precursor cells, the pygidial area – from which later the pygidium will develop, and mesodermal bands. At **48hpf** – the trochophora stage – the larva is divided into head and trunk and displays the larval eyes and three pairs of chaetal sacs with their bristles. The stomodaeum is closed and the ventral plates have fused in the ventral midline.

Abbreviations: lipid droplet, ld; prototroch, pt; ventral plate, vp; mesodermal bands, mb; stomodaeal precursor cells, spc; stomodaeum, s; pygidial area, pa; chaetal sacs; ch.s; larval eye, le; frontal bodies, fb; hours post fertilization, hpf; anterior, a; posterior, p; ventral, v and dorsal, d. *Colour usage:* ventral plate – brown, prototroch – yellow, mesodermal bands – pink, stomodaeum and spc – purple, pygidial area – green, and chaetal sacs – orange.

1.5 Aim of this thesis

In this study, I investigate the establishment of the germline in *Platynereis dumerilii*. To achieve this, I first determined the origin and development of the primordial germ cells in this polychaete, which has not been described so far. I used molecular and histochemical tools to identify a number of genes in *Platynereis*, which are known to play important roles in development of the germline in other organisms. These molecular tools should allow me to follow the development of the germ cells throughout the life cycle of *Platynereis* and address the question whether the specification of the germ cell in this specie is achieved by

the asymmetric inheritance of maternal determinants or by extrinsic signalling. Secondly, I investigate the link between the "germline genes" and axis formation, by comparing my results with the data available from other species.

2 RESULTS

The germ cells formation and their determination mechanism are tightly linked to the establishment of polarity in various species. Several genes have been described to play a role in both mechanisms. For the polychaete *Platynereis dumerilii* neither of these processes have been investigated so far. I have studied some of these genes in this worm to elucidate their function and at the same time clarify the ancestral feature of the respective gene of interest throughout Bilateria. A first step in elucidating the function of a particular gene of interest was the analysis of its expression pattern. After cloning the gene of interest, *in situ* hybridization was performed on *Platynereis dumerilii* larvae and adult worms. In this chapter, the expression patterns of several genes are reported and are compared with orthologs in other species (further below).

2.1 Germ cell specific genes

Sexual reproduction is a fundamental means of reproduction in uni- and multicellular organisms. The task to convey genomic information from two parental organisms to the progeny has been allocated to the gametes. In several well-studied organisms, such as *C. elegans* and *Drosophila*, gametes are derived from **primordial germ cells** (PGCs) that arise during early embryogenesis in a separate lineage divergent from the somatic tissue. In other organisms, for instance in mammals, primordial germ cells are produced from somatic stem cells. In either case, eventually the primordial germ cells become unipotent, or in hermaphrodites, bipotent stem cells that migrate into the developing gonads and proliferate producing large numbers of oocytes or spermatocytes respectively, which will undergo meiosis and differentiate into mature gametes (Lin, 1997; Saffman and Lasko, 1999).

In the annelids and especially in the polychaete *Platynereis dumerilii*, data on the origin and development of the germ line are incomplete. Gametogenesis has been well studied for this annelid using histological and cytological techniques (Fischer, 1975; Fischer and Dorresteijn, 2003; Hauenschild and Fischer, 1969). On the other hand, the origin of these cells is not known and no gonadal structures have been described for *Platynereis dumerilii*.

Several molecular factors used to specify germ cells have been conserved throughout Bilateria. Specific genes are expressed (transcript or/and protein) in primordial germ cells (PGCs). I will focus on *vasa*- and *nanos*- related genes, because both genes are involved in

germline development in different species. Moreover, *nanos* is a good candidate to unravel an ancestral link between germline development and early axis formation, since this was demonstrated in *Drosophila melanogaster* (Forbes and Lehmann, 1998; Gavis and Lehmann, 1994) and strikingly it is also defining the posterior end in the cnidarian Podocoryne (Torras et al., 2004).

2.1.1 Vasa is a germline marker and is expressed in not terminally differentiated cells

Vasa belongs to the DEAD-box protein family of RNA helicases. The function of Vasa has been demonstrated in the fly; it is required for the progression of oogenesis and for pole plasm assembly (Hay et al., 1988; Lasko and Ashburner, 1988; Liang et al., 1994; Schupbach and Wieschaus, 1986), and it has been suggested to be involved in translational regulation (reviewed by Johnstone and Lasko, 2001). It has been used as a germ-line specific marker in several species. I investigated the *vasa* homolog in *P. dumerilii*, because the identification of cells expressing it may enable tracing the germline in this polychaete.

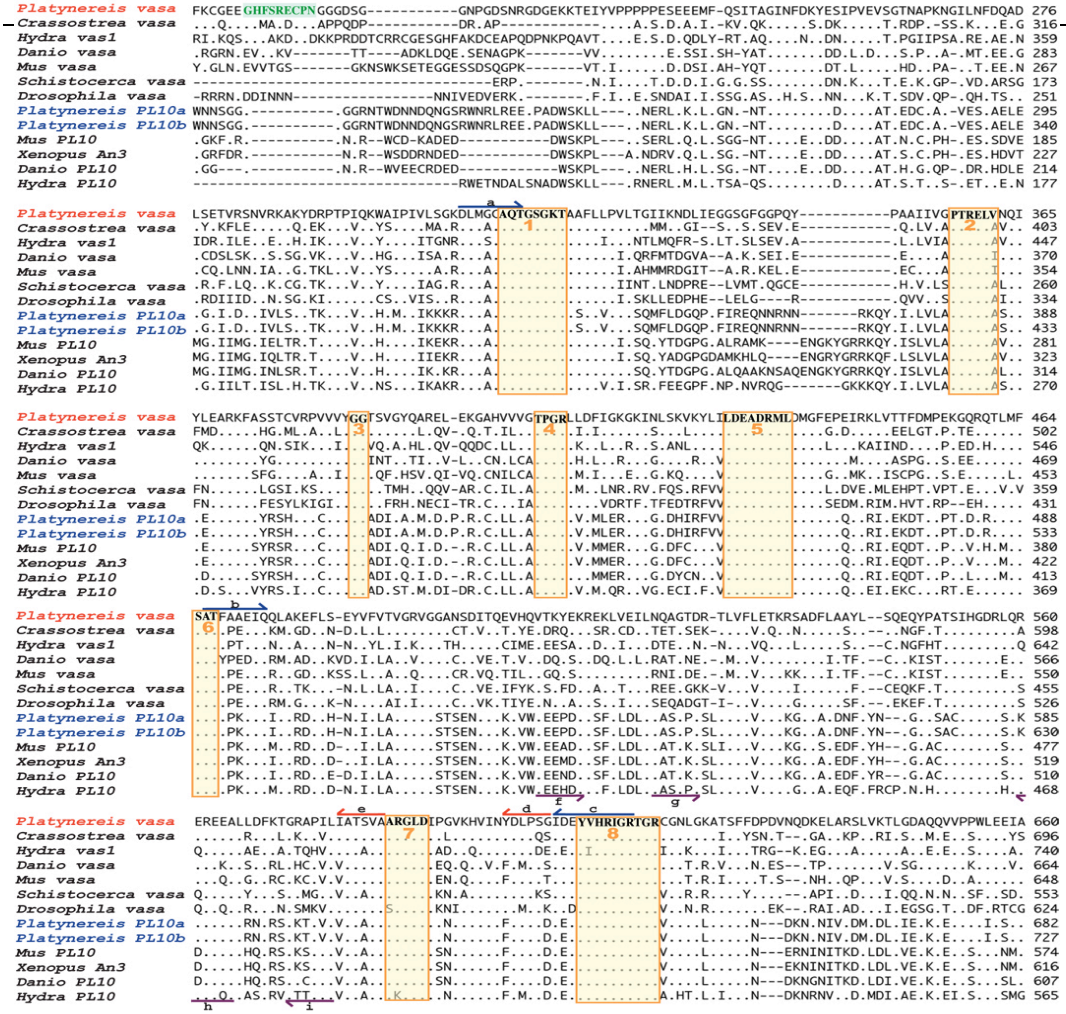
The phylogenetic tree in Figure 5B shows that *Platynereis* vasa (red) is a true ortholog of the *vasa* subfamily among the DEAD box RNA helicases. I cloned another member of the DEAD box RNA helicases in this polychaete, *Platynereis* pl10 (blue). This gene will be described later in this work.

The *Platynereis dumerilii vasa* fragment (*Pdu-vas*) is 2945bpbp long (including the 3' UTR); its open reading frame (ORF) stretches from position 104 to 2239. The ORF codes 712 AA, containing three CCHC-type zinc finger domains at the N-terminus, a DEAD box and a RNA Helicase C domain. The position of each domain is depicted in Figure 6. It is likely, according to the data obtained from the Northern and Western blots and *Platynereis* genome sequence that nearly 1kb of *Pdu-vas* codes for two additional CCHC zinc fingers and this sequence is still missing.

A theoretical molecular weight (MW) of 74.13kDa is calculated from the AA-sequence, predicted on the basis of the open reading frame. However, this value does not conform to the MW derived from the Western blot made by Nicole Rebscher with a polyclonal peptide antibody produced against a part of the *Platynereis* vasa protein (see sequence in Figure 5A, green). This antibody recognized a band around 100kDa in size. Similarly, the Formosa antibody against the grasshopper vasa homolog (Chang et al., 2002) recognized a

band around the 110kDa (Figure 14). Therefore, I presume that the size of *Platynereis* vasa protein is 100 to 110kDa.

After cloning a 1200bp fragment that covered most of the 5' end of the gene, I performed *in situ* hybridizations (ISH) on different stages of *P. dumerilii* larvae and adult worms. This was complemented by semi-quantitative RT-PCR (RT-PCR is a more sensitive method compared to ISH) and Northern blot results, which yielded information on stages, in which ISH signal was not possible to obtain, or was not obvious enough compared to the negative control. In general, the egg jelly appears to render staining difficult, as in the case of unfertilized eggs and females ready to spawn (the oocytes inside the worms release the egg jelly due to impact produced by PFA in the fixative solution, which is the first step in the ISH procedure). Males were also difficult to stain with ISH.



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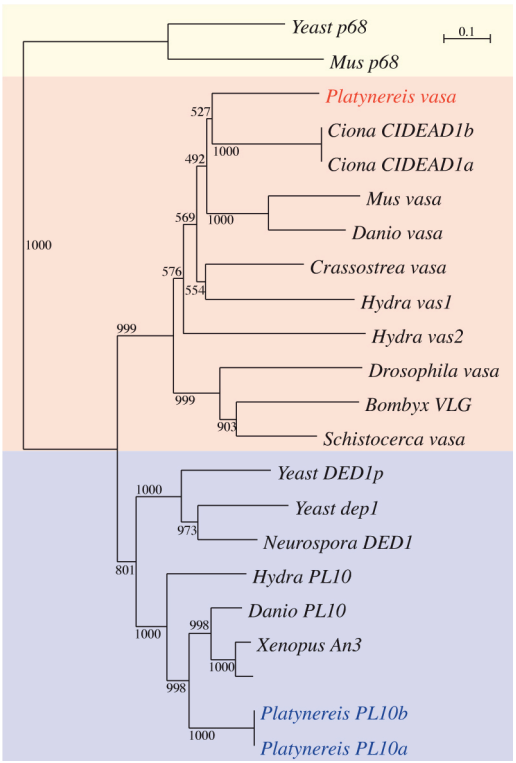


Figure 5. Vasa and pl10 amino acid sequence alignment and graphical representation of phylogenetic analysis.

(A) Sequence alignment was realized with CLUSTAL X. Decoration: Hide (as '.') residues that match *Platynereis* vasa exactly. Eight conserved motifs are enclosed in a box and numbered (1: AQTGSGKT, 2: PTREL(V/A), 3: GG, 4: TPGR, 5: LDEAD(R/E)ML, 6: SAT, 7: ARGID and 8: Y(V/I)HRIGR(T/G)GR). Degenerated primer regions marked with blue, specific primers with red for *Platynereis* vasa and purple for *Platynereis* pl10. The orientation of each primer is indicated by half arrow. AA sequence used to generate polyclonal antibodies in rabbit is highlighted in green. (B) The phylogenetic tree was constructed using the Neighbour Joining method. Bootstrap values (numbers 1 to 1000) are indicated at branch points. The branch length correlates with the evolutionary distance by counting the exchange rate since the last bifurcation (unit: exchanges per 100 positions). The vasa subfamily is marked in red and *Platynereis* vasa is highlighted. Pl10 subfamily is marked in blue, with the two Pl10 splicing variants for *Platynereis dumerilii* highlighted. Members of the p68 subfamily were taken as outgroup, and are marked in yellow. a-e, primers for race (see materials and methods).

Figure 6. *Platynereis dumerilii* vasa protein.

Platynereis vasa protein is 712 AA long and contains tree zinc finger domains at the N-terminus (green), a DEAD box domain (red), and a Helicase C domain towards the C-terminus (yellow). The position within the protein of each domain is depicted with numbers, on the right. The AA sequence was blasted against the protein families' database of alignments and HMMs (Pfam).

Pdu-vas mRNA was detected in early stages with ISH (Lartillot et al., 2002a). *Pdu-vas* mRNA was found to be ubiquitous distributed in all blastomeres, starting from 2- to 29-cell stages (Figure 7). A negative control was included to rule out any possible background staining (see Figure 7, pictures on the left). The negative control consisted in adding a sense mRNA, which should not hybridize with any transcript. The drawings in Figure 7 were made after Wilson (1892), who described the cell lineage of *Nereis*, a species closely related to *Platynereis*. The last drawing in Figure 7 represents 15hpf or prelarva stage, where the transcript was abundant in the hyposphere (at this stage it comprises the ectomesoderm) and at lower levels at the dorsal surface of the episphere. Different views of 15hpf are shown in Figure 8A-C. As mentioned before, *vasa* transcript at this stage were confined mainly to the posterior/vegetal pole of the larvae (hyposhere), suggesting degradation of the transcript in other areas, or translocation of it to the posterior. At 24hpf (Figure 8D-F) higher levels of *Pdu-vas* were detected at the most posterior end of the embryo.

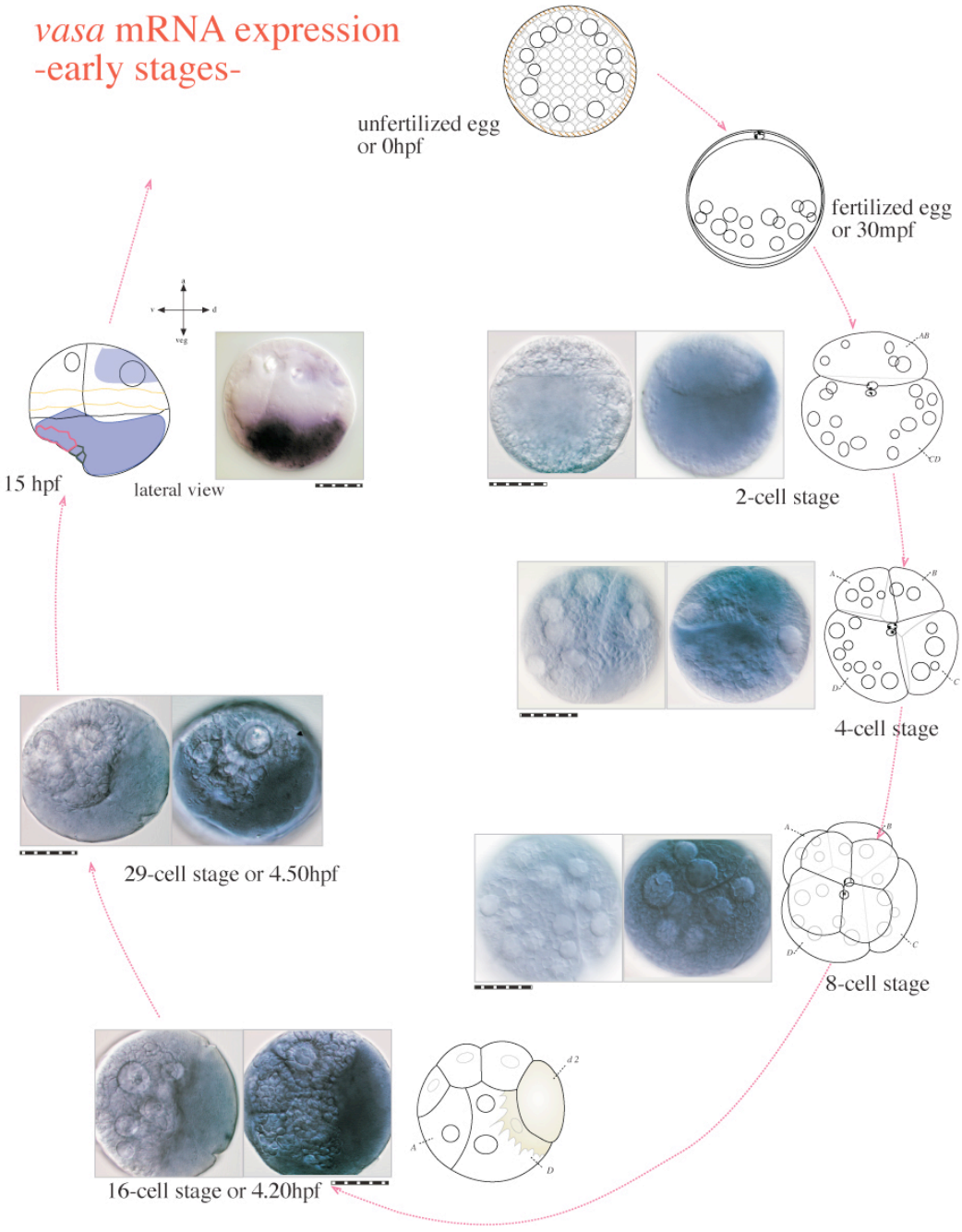


Figure 7. *Pdu-vas* mRNA expression in early stages

Schematic representation and pictures of *Platynereis dumerilii* early development and *Pdu-vas* mRNA expression from 2-cell stage to 15hpf. *Pdu-vas* is evenly distributed throughout the whole embryo from 2- to 29- cell stage, for these stages see negative control, on the left. At 15hpf, expression in the superficial dorsal region of the animal episphere and in the ectomesoderm can be detected (see Figure 8A-C for a precise description of stage 15hpf). These results are in good agreement with the outcome of the semi-quantitative RT-PCR in Figure 12. Scale bar: 80µm.

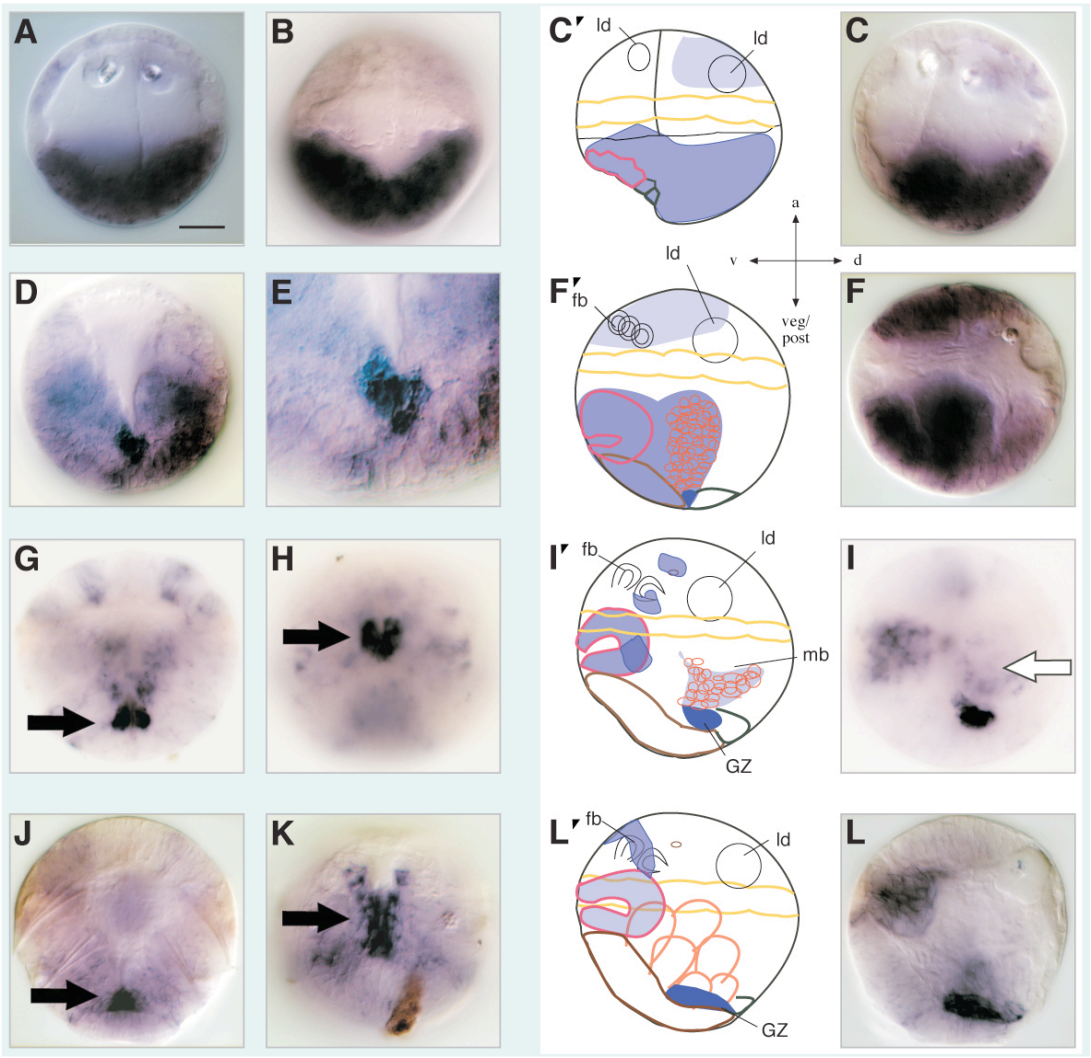


Figure 8. *Pdu-vas* mRNA expression in *Platynereis dumerilii* embryos of 15 to 48hpf and corresponding schematics in lateral view.

15hpf: (A) dorsal view, (B) ventral view and (C) lateral view and schematic, *vasa* transcript is found in the superficial dorsal region of the episphere, and in the ectomesoderm (including spc and pa). 24hpf: (D) and (E) posterior dorsal view, (E) is enlarged to show that the forming posterior GZ has significantly stronger mRNA expression. (F) lateral view, mRNA expression comprises the ventral posterior head region, mesodermal bands and stomodaeal area. 36hpf: (G) dorsal view, faint staining in the head and mesodermal bands, stronger staining in the posterior GZ, (H) posterior view focusing on V shaped staining of the posterior GZ comprising 6 to 8 cells, and (I) lateral view, *vasa* mRNA expression weakens in the stomodaeum and mesodermal bands, but remains strong in the posterior GZ which is located adjacent to the pygidial area, behind the ventral plate. 48hpf: (J) dorsal view and (K) posterior view show a pyramid shaped and elongated structure, located in the pa behind the ventral plate as depicted in (L). The drawing in (L) summarizes the superficial staining in the ventral region of the head, a weak staining in the stomodaeum and a strong mRNA expression in the posterior GZ.

Colour usage: ventral plate (brown), spc and stomodaeum (purple), pygidial area (green), mesodermal bands (light pink), chaetal sacs (light orange), prototroch (yellow) and *Pdu-vas* mRNA (different shades of blue). *Abbreviations:* apical, a; vegetal, veg; posterior, post; hours post fertilization, hpf; days post fertilization, dpf; lipid droplets, ld; frontal bodies, fb; pygidial area, pa; mesodermal bands, mb; stomodaeum, s; stomodaeal precursor cells, spc; ventral plate, vp and growth zone, GZ. *Scale bar:* 50µm for all pictures in this figure, except for E. Schematics of correspondent stages are represented with apostrophe (‘).

A posterio-dorsal view reveals a diamond shaped structure, formed by 6 to 10 cells. There was still *Pdu-vas* mRNA on slightly lower levels in the rest of the embryo, including brain cells. At 36hpf the transcript began to disappear, remaining only in the stomodaeum (Figure 8I) as heart shaped form (Figure 9C), at low levels in the mesodermal bands (Figure 8I, white arrow) and at higher levels in 6 to 8 cells in the pygidial area dorsal to the neural plate (Figure 8G-I, black arrow), which formed the posterior growth zone, GZ. At this stage *Pdu-vas* was also detected in the brain, starting in the larval eye area (Figure 9A), continuing laterally towards the ventral surface of the brain (Figure 9B) and ventrally below the prototroch (Figure 9D). The next stage that I investigated was 48hpf, the expression pattern of *Pdu-vas* here was similar to the one described for 36hpf, with some slight differences. At 48hpf the staining in the stomodaeum was noticeable weaker (Figure 8L and Figure 9E). Cells from the growth zone proliferated giving rise to a pyramid shaped structure (Figure 8J-K) located dorsal to the neural plate, right adjacent to the developing pygidium, in the middle of the 3rd segment. The staining in the brain remained ventrally on the surface, while the staining detected at 36hpf below the prototroch, moved towards the posterior (Figure 9F). At 72hpf the transcript was localized to the most distal region of the stomodaeum, which means the most dorsal part of the stomodaeum, exactly in the region where the gut changed direction from ventro-dorsal and started elongating to the posterior (Figure 10A-C, grey arrow), in some cells in the midline reaching the posterior growth zone (Figure 10A), and in the GZ (Figure 10A, B and C, black arrow), which was located at this stage between the parapodia of the 3rd segment, above and just adjacent to the pygidium. A 5dpf old worm developed jaws and was able to feed; the food in the gut often gave background staining with the WMISH procedure. Staining was slightly more difficult at later stages, because worms from 5dpf onward fed and the food in the gut often gave background staining when using WMISH procedure. *Pdu-vas* in 5dpf old worm was found in the posterior of the worm, forming a ring around the anus (Figure 10G). Looking at this structure from a ventral view, the structure resembled a butterfly (Figure 10F, black arrow). This structure is the growth zone, known as the source of the future trunk segments for all polychaete trochophores. The growth zone is a ring of ectoteloblasts enclosing a pair of M-cells, just in front of the telotroch (Anderson, 1973). In *P. dumerilii* this structure is located above the pygidium, posterior to the junction of the 3rd pair of parapodia in 5dpf old worms, and was expressing *Pdu-vas* mRNA (Figure 10E). *Pdu-vas* mRNA was

diminished after 6dpf; the transcript was no longer detectable either with the WMISH, RT-PCR nor northern analysis (see Figure 12 and Figure 13). Vas protein was present at 36hpf in the growth zone persist after 6dpf (N. Rebscher, personal communication and discussed later).

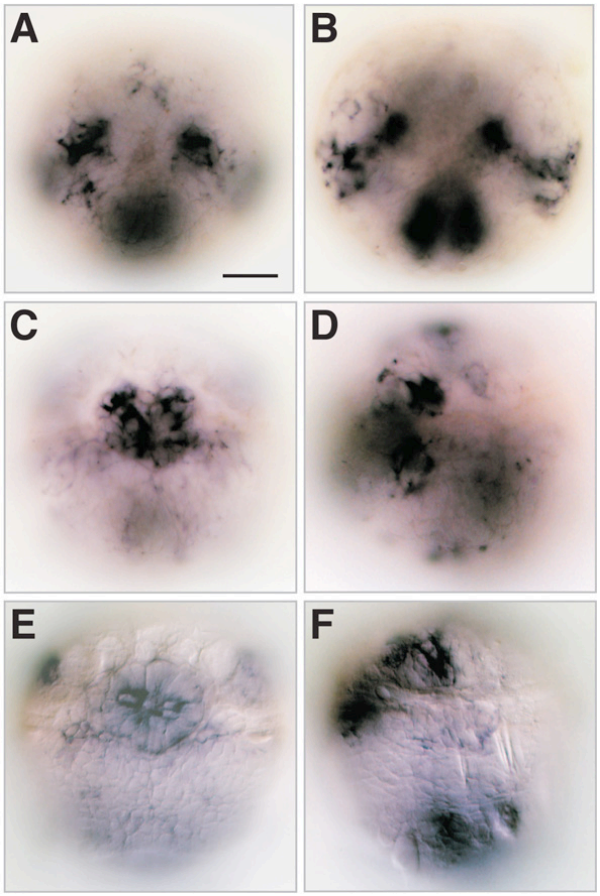


Figure 9. *Pdu-vas* mRNA expression in *Platynereis dumerilii* embryos of 36 to 48hpf.

36hpf: (A) superficial anterior view, *vasa* mRNA in the larval eye area, (B) a deeper anterior view shows the continuity of the staining towards the ventro-lateral region of the brain. (C) ventral view, heart shaped structure in the stomodaeum. (D) lateral superficial view showing strong mRNA expression on the ventral half of the embryo, staining above and below the prototroch. 48hpf: (E) ventral view depicting reduced expression in the stomodaeal area. (F) lateral superficial view showing a continuous superficial staining on the ventro-lateral side of the brain. Scale bar: 50µm (includes all pictures). *Abbreviation:* hours post fertilization, hpf.

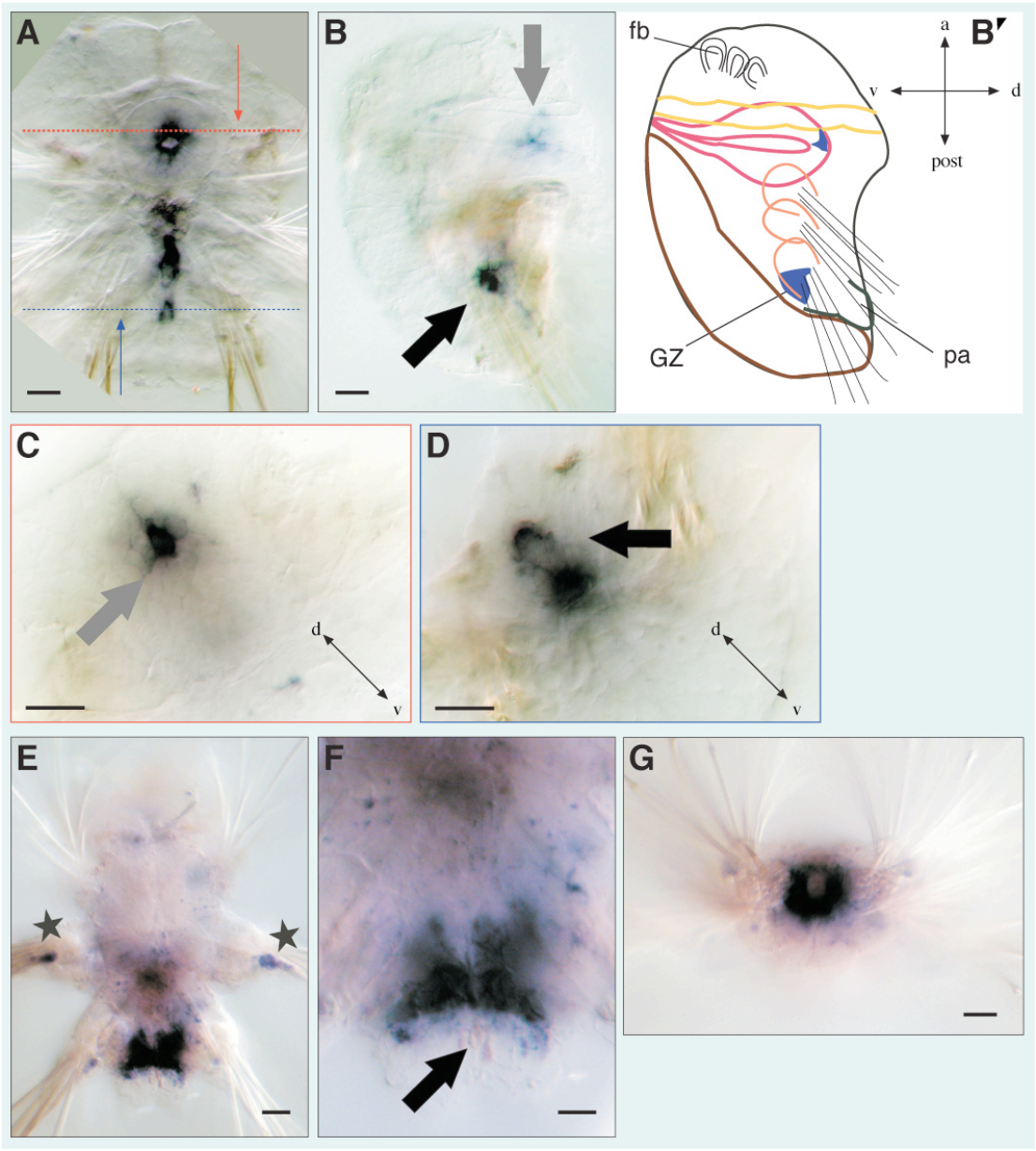


Figure 10. *Pdu-vas* mRNA expression in *Platynereis dumerilii* young worm of 72hpf and 5dpf.

72hpf: (A) ventral optical section shows mRNA expression in the distal stomodaeum and in the deep midline. (B) lateral view, expression in the distal part of the stomodaeum and accumulation of mRNA in cells located between the 3rd segment, in the pygidial area and dorsal to the ventral plate, the posterior GZ (C) anterior view of stomodaeal staining, and (D) posterior view of the posterior GZ. 5dpf: (E) ventral view showing a butterfly shaped structure in the pa, enlarged in (F) and background staining in parapodial gland cells (asterisks). (G) posterior view of the butterfly shaped structure forming a ring around the developing hindgut.

Colour usage: ventral plate (brown), stomodaeum (pink), pygidial area (green), chaetal sacs (light orange), prototroch (yellow) and *Pdu-vas* mRNA (different shades of blue). *Abbreviations:* anterior, a; posterior, post; dorsal, d; ventral, v; hours post fertilization, hpf; days post fertilization, dpf; frontal bodies, fb; pygidial area, pa; stomodaeum, s; and growth zone, GZ. *Scale bar:* 50µm. Schematic of correspondent B is represented with apostrophe (‘).

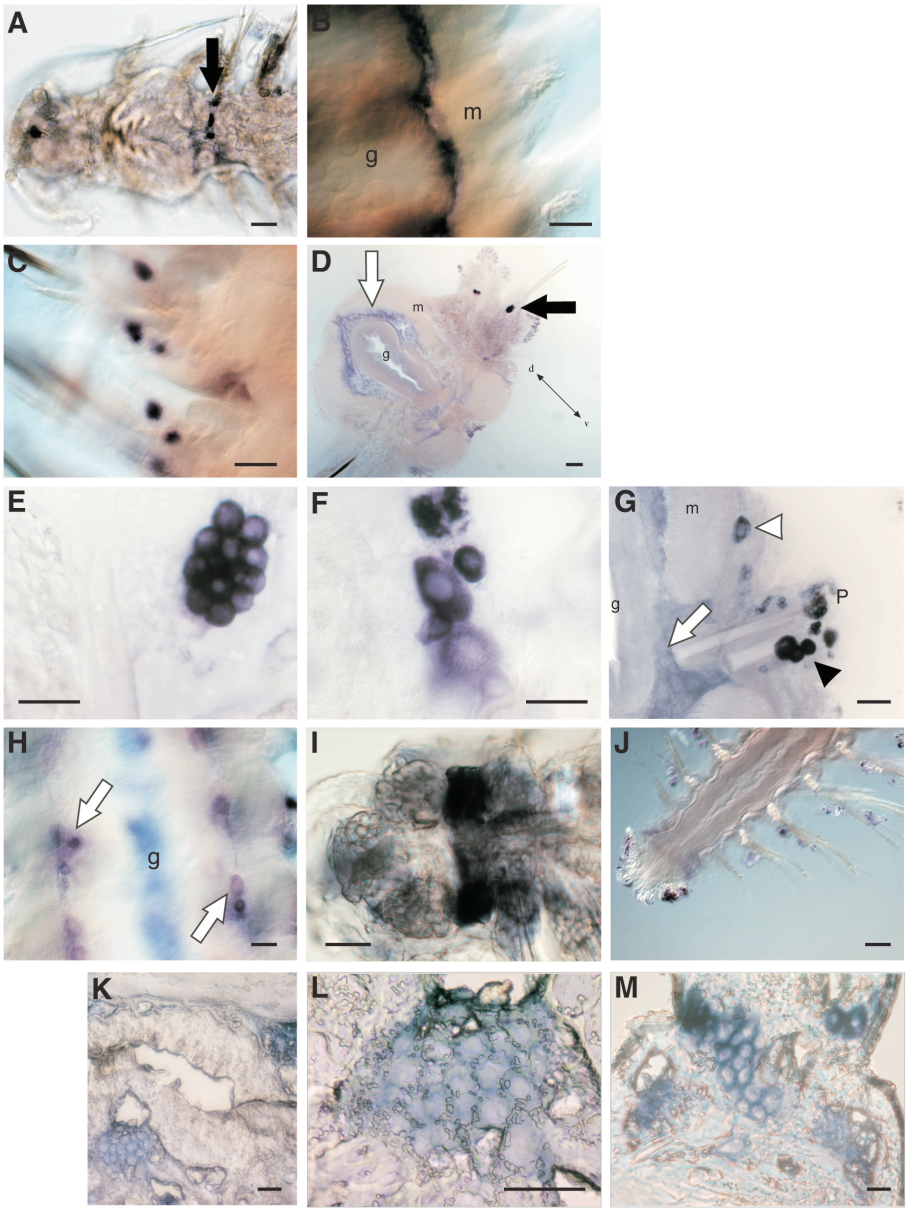


Figure 11. *Pdu-vas* mRNA expression in *Platynereis dumerilii* worms

(A) groups of cells forming first a dorsal stripe and then a ring specifically around the 4th to 5th segment of a juvenile worm-black arrow. The staining is not detectable anymore in worms entering epitoky. (B) *Pdu-vas* mRNA in the coelom of anterior segments. (C) *Pdu-vas* mRNA accumulation in groups of cells in the parapodia of some worms older than 1 to 1 1/2 months. (D) transversal section of a worm of 42 segments, *Pdu-vas* in the coelom, in the region between the gut and muscles-white arrow, and in gonial clusters in the parapodia, enlarged in (E). (E) gonial clusters of 14 to 16 cells expressing *Pdu-vas*. (F) previtellogenic oocytes located in the coelom. These latter cells are located in the coelomic cavity along the gut, as shown in (H-white arrows). (G) *Pdu-vas* mRNA detected in oocytes floating in the coelomic cavity- white arrow, in the parapodia- black arrowhead, and between the muscles and the ectodermal layer-white arrowhead. (I) tail region of a 2 months old worm of 20 segments, *Pdu-vas* in the GZ. (J) tail region of a worm with 47 segments, where no *Pdu-vas* is detected. (K), (L) and (M) are paraffin longitudinal sections of a worm showing gonial clusters along the gut (K), enlarged in (L) and in the parapodia (M). Scale bar: 50μm, except for A, B and C, which is 100μm. Abbreviations: gut, g; muscles, m; parapodia, P.

In worms older than 6 weeks (30-36 segments long), groups of *Pdu-vas* expressing cells were found on the dorsal side, and then in a ring-like structure around the 4th to the 5th segment (Figure 11A). In the following segments this staining was absent, but I observed a staining in the peritoneum (Figure 11B and D, white arrow), which is the epithelial wall of the coelomic cavity. Groups of cells in the parapodia expressed *Pdu-vas* (Figure 11C and D, black arrow). Each of these groups of cells contained 14 to 16 cells, with each cell having a diameter of 5 to 7 µm. The cluster might conform to the oogonial cluster designated by Fischer (Fischer, 1975) as young oocytes in stage 2 to 3 (the staining by ISH did not allow to distinguish between these two stages). Each cell of the cluster had a spherical, enlarged nucleus, so that in sections the cytoplasm appeared as a rather narrow belt expressing *Pdu-vas*, as shown in Figure 11E. According to Fischer (Fischer, 1975), these clusters were surrounded by a sheath of cells, which served as a physical support rather than having a nutritive function, and were floating in the coelomic cavity. I found these cells in the parapodia (see Figure 11D, black arrow, and M) and sometimes between the muscles and gut (see Figure 11K and L). The coelomic fluid occupied all these regions and gonial clusters or/ and oocytes could float into these areas. Oocytes expressing *Pdu-vas* were found along the gut filling the coelomic cavity (Figure 11G, white arrow) these cells had a big round nucleus with a diameter of 50 - 70 µm (see Figure 11F, G and H). Oocytes were also found in all places where the coelomic fluid could be displaced, as in the parapodia (Figure 11G, black arrowhead) and between the dorsal muscles and ectoderm layer (Figure 11G, white arrowhead). Two months old worms (15 to 36 segments long) showed a strong *Pdu-vas* positive staining in the growth zone (Figure 11I). In worms longer than 38 segments no *Pdu-vas* positive cells were detected in the growth zone region (Figure 11J).

To estimate the levels of *Pdu-vas* transcript throughout the development of this polychaete and to confirm the results obtained by WMISH, I performed semi-quantitative RT-PCR. *Pdu-actin* was used as a reference control for total mRNA. The results of the RT-PCR are shown in Figure 12, for the sequence of the primers see chapter 4.11.3.

Pdu-vas transcript (represented by a 361bp fragment in Figure 12) was abundant in unfertilized eggs and significantly decreased at 72hpf, possibly due to degradation of the maternally contributed mRNA, or due to decrease of zygotic gene transcription. In 5 day-old worms, the level of the transcript increased, and later in juveniles of 25-30 segments

(22 to 50 days old) it was almost undetectable, as well as in males entering epitoky. There was no expression of *Pdu-vas* mRNA in males that were ready to spawn, in contrast to females, where *Pdu-vas* was detected in females entering epitoky, and in females that were ready to spawn. I used *Pdu-actin* as a quantitative reference indicating the quantity of total mRNA for each stage. Unfertilized eggs (because they are unicellular) contained less actin compared to other stages, therefore the actin band was weaker in the control of this stage (see Figure 12, 222bp fragment).

In order to verify the results of both WMISH and RT-PCR and to estimate the size of the transcript including the UTR region, total RNA from different developmental stages (8 μg per lane, except for juveniles with just 3,8 μg) was subjected to Northern blot according to standard procedures (see chapter 4.9). To rule out that total RNA for juveniles was extracted from a mixture of females and males, RNA of one single juvenile was used. Note that the quantity of RNA was less than half of what was loaded for the other samples. Juveniles of 20-25 segments have few RNA when compared to other stages. *Pdu-vas* transcript was 5400bp in size (Figure 13, red arrow), and it was detected in unfertilized eggs, decreased in quantity at 48hpf and was present again in 5dpf old worms. No transcripts were visible in males, but were abundantly present in females (Figure 13). Females ready to spawn (fully matured) showed a "fuzzy" higher band both at the level of the transcript as at the 18S ribosomal RNA (rRNA) subunit, which runs at the level of 2kbp (Figure 13, black asterisks). This might be due to some proteins - probably glycoproteins – found in these females that might cause problems in extraction and gel-electrophoresis.

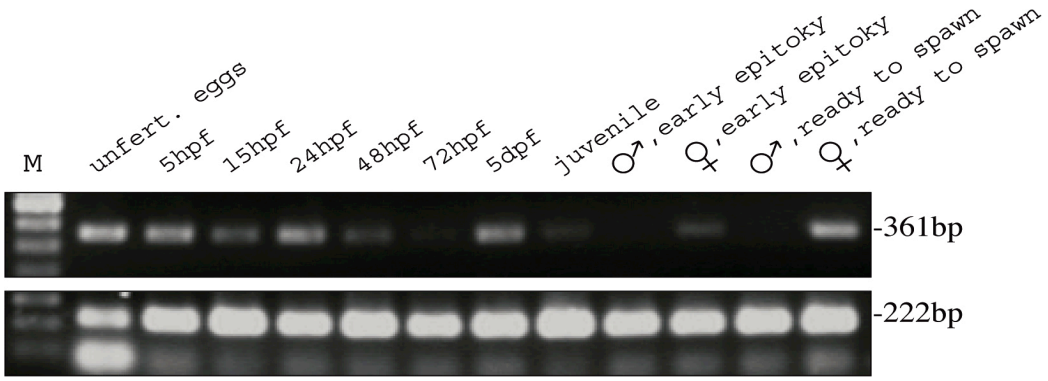


Figure 12. RT-PCR of *Pdu-vas* in different stages of development.

Pdu-vas transcript (361bp fragment) is abundant in unfertilized eggs and females, which are ready to spawn. It significantly decreases at 72hpf. Levels in males entering epitoky are almost undetectable, and there is no expression of *Pdu-vas* mRNA in males that are ready to spawn. A control with *Pdu-actin* is included in the picture below (222bp fragment). "M" stands for the DNA ladder marker. Primers used: for vasa, *race_vasa_up1* and *race_vasa_lo2*; for actin, *actin_fw2* and *actin_rev2*.

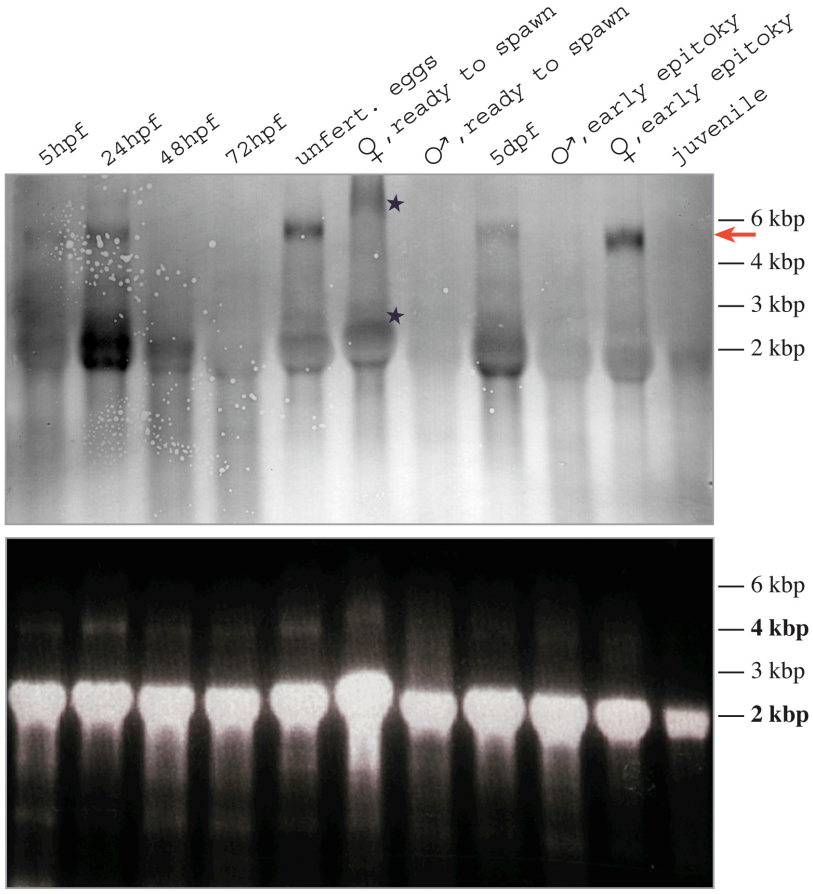


Figure 13. Northern analysis of *Pdu-vas* in different developmental stages of *P. dumerilii*

Pdu-vas mRNA is 5400bp (red arrow). It is detectable in unfertilized eggs, drops and is undetectable at 48hpf. It is present again at 5dpf. No *Pdu-vas* mRNA is detectable in juveniles, nor males. The transcript is again visible in female worms entering epitoky and a bigger "fuzzy" band is observed in females that are ready to spawn. The "fuzziness" is due to a slower migration of this sample; two black asterisks show the shift, both at the level of the transcript as at the 18S ribosomal RNA (rRNA) subunit, which runs at the level of 2kbp. The picture of the total RNA is included below as quality control, where the band at 4kbp represents the 28S, and at 2kpb the 18S rRNA subunit.

The expression level, and localization of *Pdu-vas* mRNA have been elucidated for several stages. To get insight into the *Platynereis* vas protein localization I performed immunohistochemistry in *Platynereis* larvae and Western blot with the polyclonal Formosa antibody raised against the vasa protein of the grasshopper (Chang et al., 2002), and which cross-reacted with *Drosophila* vasa protein (Dm-Vas) as well. It was therefore likely that it also would recognize the *Platynereis* vasa protein. Indeed, this antibody (1:500 dilution) recognised a single band at 110kDa in *Platynereis dumerilii* protein extract of a single batch of 24hpf (Figure 14A, first line and red arrow). Two positive controls were included,

the second lane contained reticulosites from a construct containing GST- tag and part of *Drosophila melanogaster* vasa (Dm-GST-Vas), the third lane contains part of *Drosophila melanogaster* vasa protein expressed in bacteria, and purified (Figure 14A). I detected clear staining in a group of cells that are located dorsal to the ventral plate, in the posterior growth zone (Figure 14B-D).

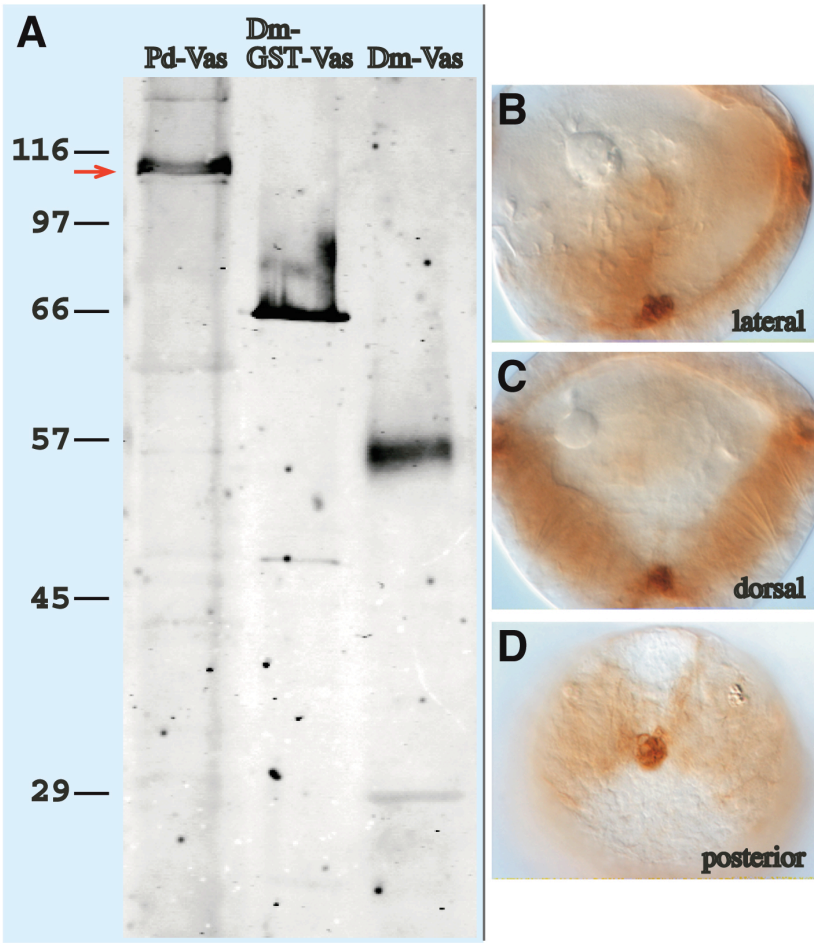


Figure 14. Western blot and immunohistochemistry staining with Formosa antibody

(A) Western blot –antibodies dilution 1:500-, a single band, likely to correspond to Pdu-Vas at ~110kDa (red arrow and first column). As positive control: Dm-GST-Vas construct at 65kDa (middle column), and after the GST tag has been removed at 56kDa. Formosa antibody recognised both Pdu-Vas and Dm-Vas. 50µg of protein extract loaded per lane. Molecular weight Marker in kDa, numbers on the left. 48hpf (1:30 dilution): (B) lateral view, Pdu-Vas localized in a group of cells behind the ventral plate, in the pygidial are, the GZ. (C) dorsal view, Pdu-Vas in the pygidial area. (D) posterior view showing Pdu-Vas in the same group of cells.

Abbreviations: GZ, growth zone; Pdu, *Platynereis dumerilii* and Dm, *Drosophila melanogaster*.

The polyclonal Formosa antibody was a kind gift from Michael Akam, but unfortunately the quantity was not enough to achieve a better signal in other developmental stages of this polychaete. Therefore, new polyclonal peptide antibodies were raised in rabbits and the sequence used for its production is highlighted in green in Figure 5A. These antibodies recognised a band at around 100kDa and cross-reacted with vasa protein in *Platynereis*

dumerilii (N. Rebscher, personal communication). This data will be discussed later in this work.

2.1.2 *Pl10 expression in Platynereis*

Vasa- and pl10-related proteins are very similar to each other, see alignment in 6.1.1. They share eight characteristic sequence motifs, including the DEAD (Asp-Glu-Ala-Asp) box (Linder et al., 1989). *Platynereis* pl10 and *Platynereis* vasa also contain these 8 conserved motifs highlighted in yellow in Figure 5A. In mouse, *pl10* is exclusively expressed in the male germline (Leroy et al., 1989) and it is thought that *vasa*-related genes arose form a duplication of *pl10*-related genes (Mochizuki et al., 2001). As I mentioned before, both vasa and pl10 proteins are RNA helicases. Vasa protein or/and *vasa* mRNA is used as a germline marker in most of the organisms were it has been studied. Did vasa evolve a higher specificity to the RNAs that are specifically expressed in germline when compared to pl10? Which common features do these two genes share? Thus, it was interesting to investigate which role of pl1o in the polychaete.

The phylogenetic tree in Figure 5B shows that both *Platynereis* vasa and *Platynereis* pl10 are true homologs of their corresponding subfamilies. I used p68 as outgroup, which represents another subfamily of the DEAD box RNA helicases, to construct this phylogenetic tree. *Platynereis* pl10 contains the eight conserved motifs (Figure 5A) that characterise RNA helicases belonging to the DEAD box family (Linder et al., 1989).

Pdu-pl10 has two splicing variants, and I call them *Pdu-pl10a* and *Pdu-pl10b*. *Pdu-pl10a* mRNA is 2819bp long, with an ORF that codes a protein of 772AA. *Pdu-pl10b* is 2991bp long and its ORF codes a protein of 881AA, which has a theoretical molecular weight of 89.28kDa according to http://www.ualberta.ca/%7Estothard/javascript/prot_mw.html. *Platynereis* pl10 protein has two main domains, the DEAD box and the Helicase C domain (Figure 15).

Figure 15. *Platynereis dumerilii* pl10 protein domain architecture.

The longest splicing variant of this protein (PL10B) consist in 816 AA, while PL10A lacks 45 AA (starting at position 36 to 80). The main domains are the DEAD box (green) and the Helicase C domain towards the C-terminus (red). The position of each domain is depicted with numbers, on the right. The AA sequence was blasted against the Protein families' database of alignments and HMMs (Pfam).

To get insights of the role of *Pdu-pl10*, the first step after cloning the gene was to study the localization of its mRNA with WMISH. At 24hpf its RNA expression was detectable in the whole embryo, excluding the macromeres and the prototroch; lower levels were detected in the middle brain and in the most posterior of the pygidial area (Figure 16A-C). At 48hpf the expression remained in the whole embryo at low levels, except in the stomodaeum (Figure 16D and E, white arrow), and in the region behind the ventral plate, in and posterior to the pygidial area (Figure 16E, black arrow), where its expression was stronger. At this stage, the transcript showed high levels of expression at the inner hyposphere, in the mesodermal bands and was lost in the ventral plate, as shown in Figure 16F. In 72hpf old worms, many cells ubiquitously expressed *Pdu-pl10*, clearly showing stronger expression in the stomodaeum (Figure 16G-I) and in the median region, dorsal to the ventral plate, in and posterior to the pygidial area (Figure 16G and H, black arrow). At 5 and 6dpf respectively, the transcript was almost undetectable in the head. At 5dpf, some *Pdu-pl10* positive cells were found between the parapodia of the 3rd segment, anterior to the forming gut (Figure 17a, black arrow). These cells at 6dpf might have migrated to the growth zone, or the growth zone cells were expressing 'newly' *pl10* mRNA (Figure 17B, black arrow). After 5dpf worms started to feed and sometimes a background staining is observed, caused by the gut content as shown in Figure 17B. New segments started to bud from the growth zone around 14hpf. In 14dpf old worms a strong *Pdu-pl10* expression was observed in the growth zone (Figure 17C, black arrow and D), the expression in this zone remained in 1mpf old worms (Figure 17E). At this stage, clusters of cells expressing *Pdu-pl10* were found in the coelom and in the parapodia (Figure 17F and G, white arrow). The morphology of these clusters could be better seen in longitudinal optical sections of a worm of 47 segments (Figure 18D, white arrow head, and E). These clusters were around 30 to 40μm wide, and were formed from many cells (for this case more than 16).

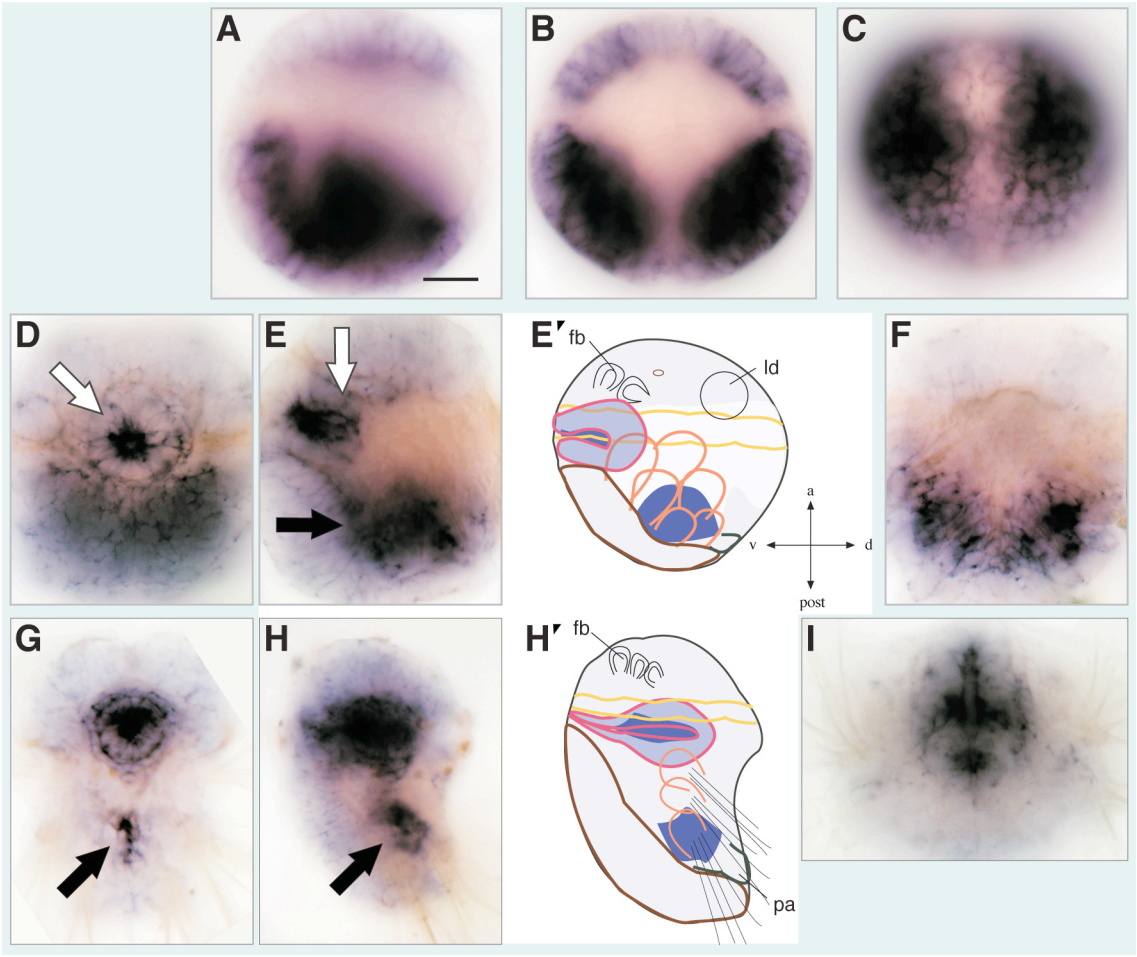


Figure 16. *Pdu-pl10* mRNA localization in 24 to 72hpf larvae and lateral view drawings of the corresponding stages.

24hpf: (A) lateral view, *Pdu-pl10* is strongly expressed in the hyposhere. (B) dorsal view, *Pdu-pl10* transcript distributed overall, excluding the middle brain, and most posterior region of the pa. Stronger expression in the mesodermal bands, as shown in posterior view represented in (C). 48hpf: (D) ventral view, *Pdu-pl10* mRNA strong expression in cells of the central stomodaeum-white arrow, and faint signal in the rest of the embryo. (E) lateral view, strong mRNA expression dorsal to the ventral plate, in and posterior to the pa-black arrow, and in the stomodaeum-white arrow. (F) dorsal view, localization of *Pdu-pl10* transcript in the inner region of the hyposhere, in the mesodermal bands. 72hpf: (G) ventral view, strong *Pdu-pl10* mRNA expression in the region within the median-black arrow. (H) lateral view, high levels of transcript in the stomodaeum and dorsal to the ventral plate, above the pygidium-black arrow. (I) optical transversal section of the stomodaeum, *Pdu-pl10* mRNA gets localized to the distal and middle stomodaeum.

Colour usage: ventral plate (brown), stomodaeum (purple), pygidial area (green), chaetal sacs (light orange), prototroch (yellow) and *Pdu-pl10* mRNA (different shades of blue). *Abbreviations:* anterior, a; posterior, post; ventral, v; dorsal, d; hours post fertilization, hpf; lipid droplets, ld; frontal bodies, fb; pygidial area, pa and stomodaeum, s. *Scale bar*: 50µm for all pictures in this figure, except for I. Schematics of correspondent stages are represented with apostrophe (‘).

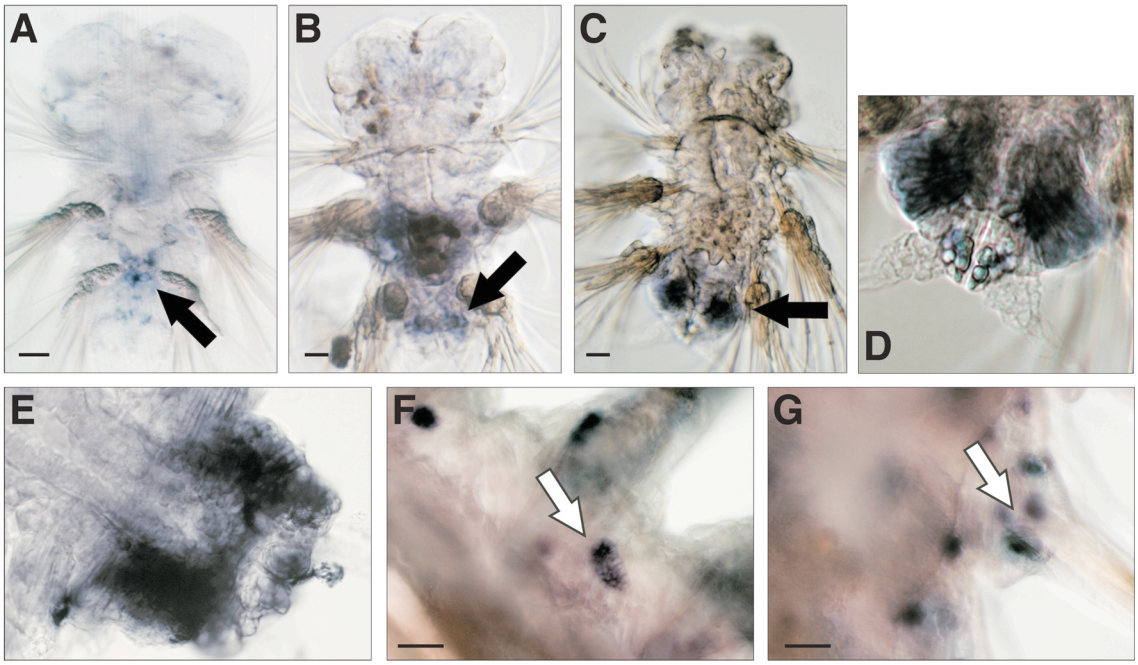


Figure 17. *Pdu-pl10* mRNA expression in young worms.

5dpf: (A) ventral view, *Pdu-pl10* mRNA in some cells to the posterior to the forming gut, between the middle region of the third segment, black arrow. 6dpf: (B) *Pdu-pl10* transcript in the growth zone, black arrow; darker staining of the gut belongs to background caused by the food. 14dpf: (C) *Pdu-pl10* transcript in the GZ, black arrow and enlarged in (D). 1mpf: (E) mRNA in the growth zone and in groups of cells within the coelom, shown in (F, with arrow) and parapodia, shown in (G, white arrow). *Scale bar:* 20µm, except for F and G, 50µm. *Abbreviations:* days post fertilization, dpf and growth zone, GZ.

In worms of 47 segments, a ring-liked structure expressing *Pdu-pl10* was detected between the 4th and 5th segment (Figure 18A), similar to the structure expressing *Pdu-vas*. The structure comprised cloudy clusters of cells as shown in Figure 18B, white arrowheads. In the following segments, *Pdu-pl10* mRNA was found in cells in the coelomic cavity, and in clusters of *Pdu-pl10* positive cells, which were located in the parapodia. In the next segments, the clusters of *Pdu-pl10* positive cells were also detected in the coelom, between the gut and the muscles (Figure 18D, white arrowhead, and E), as well as in the parapodia. In the last segments no *Pdu-pl10* expressing cells were found (Figure 18F), either in the most posterior end of the worm. Paraffin longitudinal sections of a worm of around 50 segments showed clusters of many small cells located along the gut filling the coelomic cavity; these might have been spermatogonia clusters (Figure 18G, white arrowhead). Oocytes filling the coelomic cavity of the region between the gut and muscles and of the parapodia expressed *Pdu-pl10* as shown in transversal sections of a female depicted in Figure 18H and I. The localization of the signal with WMISH in preparations of worms in

middle and late epitoky was not achieved. Nevertheless, a strong fast staining (compared to the negative control and other genes) was noticed in male worms, data not shown.

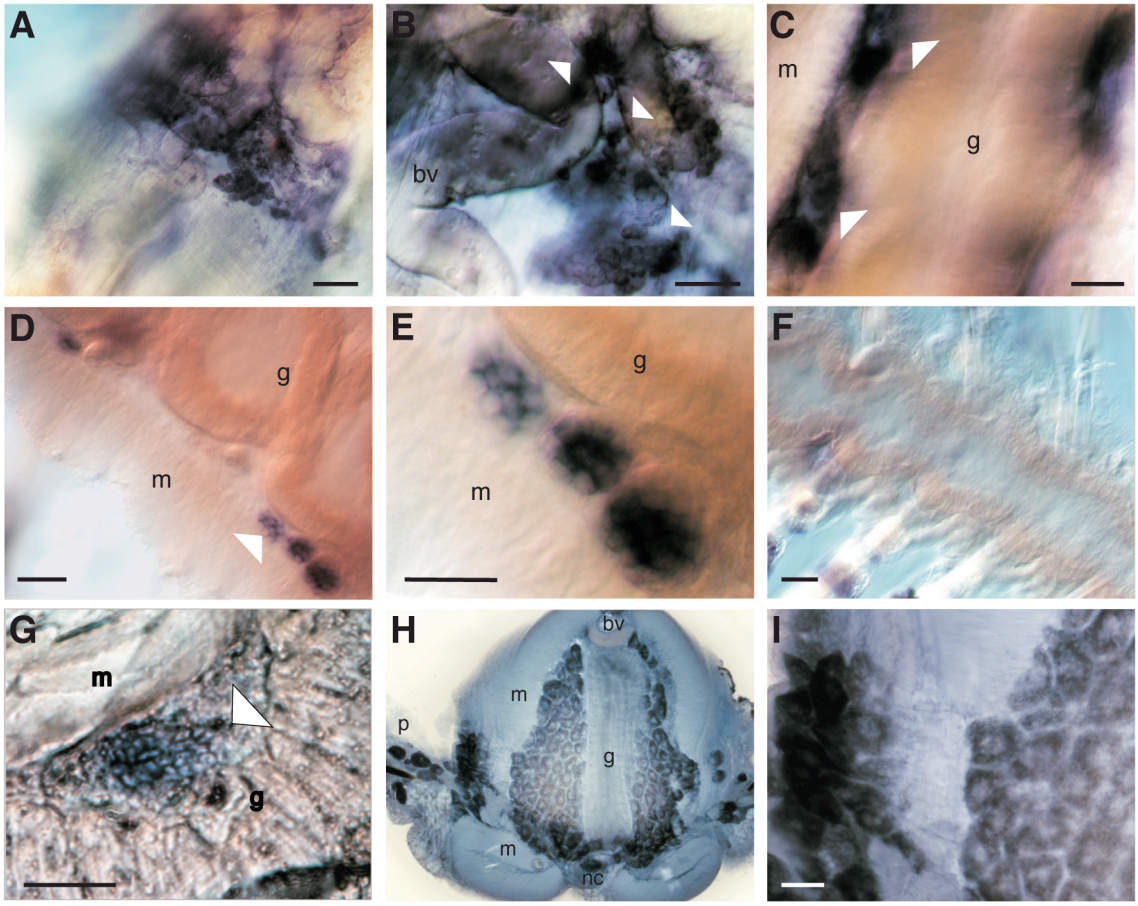


Figure 18. *Pdu-pl10* mRNA expression in worms older than one month or/ and bigger than 30 segments.

(A) *Pdu-pl10* mRNA localization in cells forming a ring around the 4th and 5th segment, enlarged in (B), where group of cells expressing *Pdu-pl10* in a cloudy form are shown - white arrowheads. (C) *Pdu-pl10* in anterior segments, located in the coelomic cavity between the gut and muscles - white arrowheads. (D) *Pdu-pl10* in cluster of cells in the coelom-white arrowheads, enlarged in (E). (F) no *Pdu-pl10* detected in the last units of a 47 segment long adult worm. (G) longitudinal paraffin section of what seems to be a spermatogonia cluster, located in the coelom – white arrow. (H) transversal section of a 55 segment long female. Oocytes, expressing *Pdu-pl10* are filling the coelom; some are found in the parapodia, others between the gut and the muscles. (I) enlarged form (H), oocytes are distinguished by its size – 50 to 70µm of diameter, its big nucleus and granular structure. *Scale bar:* 50µm. *Abbreviations:* nerve cord, nc; blood vessel, bv; parapodia, P; gut, g and muscles, m.

Semi-quantitative RT-PCR was performed to corroborate the WMISH results and to determine the level of *Pdu-pl10* expression in the different developmental stages of this polychaete. It could be seen in Figure 19 that the maternal mRNA for this gene decreased after fertilization and probably before 5dpf. *Pdu-pl10* transcript was present in all stages, although at very low levels for juveniles and in males that were ready to spawn. To confirm these results, RT-PCR was performed with 5 times more amount of template, data

not shown. In males entering epitoky and in females that were ready to spawn, *Pdu-pl10* was present at high levels.

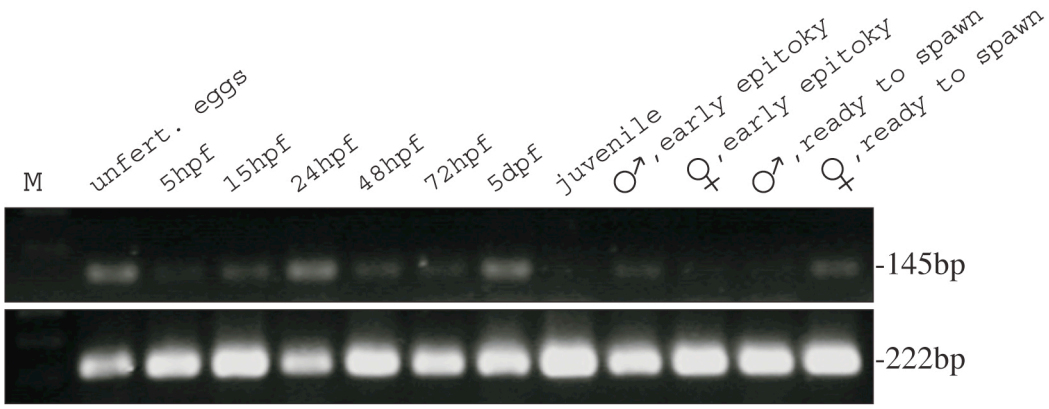


Figure 19. RT-PCR results for *Pdu-pl10*

Pdu-pl10 transcript (145bp fragment) is abundant in unfertilized eggs, males entering epitoky and females, which are ready to spawn. The transcript decreases for the first time by 5hpf. Even though it is present at all stages of development, it shows relatively low levels in juveniles and in males. A control with *Pdu-actin* is included, picture below (222bp fragment). "M" stands for the DNA ladder marker. The primers used for *Pdu-pl10* RT-PCR were race_pl10_up2 and race_pl10_lo2.

2.1.3 Pdu-vas and Pdu-pl10 expression in regenerating tails

It is known for polychaetes that they have the ability to regenerate their posterior segments (Hofmann, 1966). *Pdu-vas* and *Pdu-pl10* in the posterior growth zone in larval stages, and in the growth zone in adult worms suggested that these genes are up regulated in highly proliferating tissue. It has been reported that *vasa* and *pl10* are expressed in interstitial (i-) cells in *Hydra magnipapillata* (Mochizuki et al., 2001) and in neoblasts in *Dugesia japonica* (Shibata et al., 1999), where both kind of cells are known for their ability to give rise to various cell types (multipotency). These data suggested that these genes might play an important role in regenerative tissue; therefore I tested the expression of both genes in regenerating tails.

Pdu-vas mRNA staining in regenerating tails was quite similar when compared to *Pdu-pl10* staining. The staining was observed in all the regenerating segments (Figure 20A and C), and no staining was detected in the pygidium. One day after cutting off the tail (day post-cut, dpc) the blastema had already formed, the staining was detected in the layer below the ectoderm. This layer covered the whole blastema (Figure 20E). Newly forming

segments were visible at 4dpc. The staining in this case was localized in the innermost layer outlining the forming anus (Figure 20F) and/or the extending gut. The staining in the already existing segments of the worm differed between *Pdu-vas* and *Pdu-pl10*. *Pdu-vas* was more restricted to few cells lying between the muscles and gut (Figure 20A-green box), an example for this was depicted in Figure 20B-white arrow. In these preparations, *Pdu-vas* positive cells were detected in the peritoneum (which is the lining around the gut), in this case at the border between the "older" tissue and the regenerating zone. On the other hand, *Pdu-pl10* staining was much more broad than *Pdu-vas* staining, and it was detectable at least two segments away from the regenerating area, as depicted in Figure 20C (green box) and D.

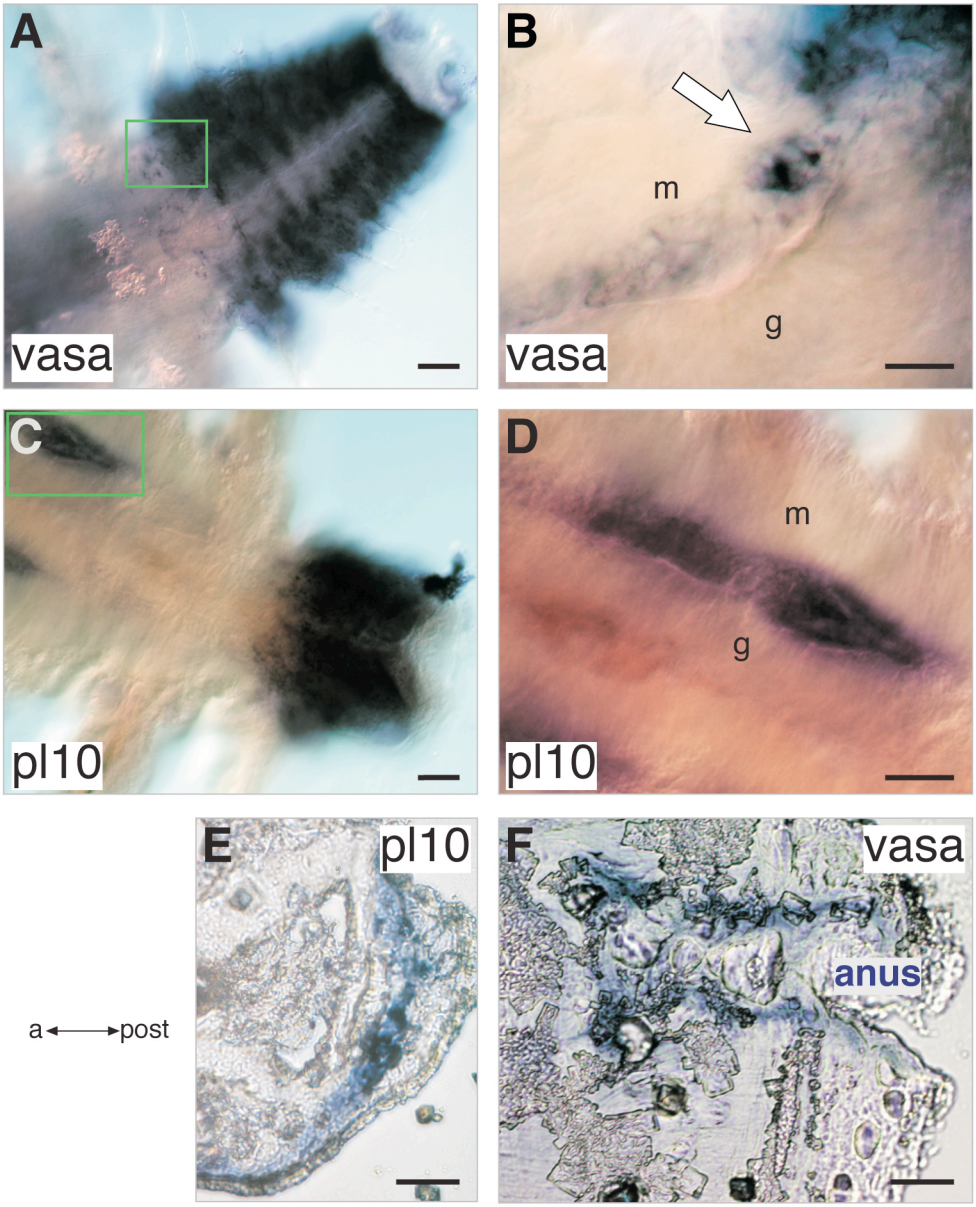


Figure 20. *Pdu-vas* and *Pdu-pl10* mRNA expression in regenerating tails.

(A) regenerating tail after 10dpc, *Pdu-vas* mRNA detected in all regenerating segments. (B) enlarged longitudinal optical section of (A- green)- deeper view, *Pdu-vas* positive cells are detected in the peritoneum, at the border between the already existing tissue and the regenerating zone-white arrow. (C) regenerating tail after 10dpc, *Pdu-pl10* mRNA localized in all regenerating segments and in cells of the peritoneum two segments away from the cut, in the same way as found in juveniles. (D) enlarged longitudinal optical section of (C-green), *Pdu-pl10* transcripts detected in peritoneal cells. (E) paraffin longitudinal section of the forming blastema after 1dpc, *Pdu-pl10* mRNA detected in the layer, which covers the whole blastema, located below the ectoderm. (F) paraffin longitudinal section of regenerating tail after 4dpc, *Pdu-vas* mRNA in the innermost layer outlining a ring around the forming anus. Scale bar: 50µm, except for B and D, which is 100µm. Abbreviations: gut, g; muscles, m; anterior, a; posterior, p and day post cut, dpc.

Thus, the expression pattern of both *Pdu-vas* and *Pdu-pl10* in regenerating tails was identical; nevertheless, a difference was noticeable in the expression pattern of already

existent segments, whereas *Pdu-pl10* mRNA is more broadly expressed, while *Pdu-vas* is localized to fewer cells.

2.1.4 Piwi, an "active morphogenesis" marker that is co-expressed with vasa and pl10

Piwi is a nucleoplasmic protein that was first described for *D. melanogaster*; it is co-localized with *vasa* and controls germline cell maintenance and division (Cox et al., 1998; Cox et al., 2000). *Piwi* homologs have been identified in *Paramecium* (Obara et al., 2000), *C. elegans*, mouse (Cox et al., 1998; Cox et al., 2000), zebrafish and human (Tan et al., 2002) (Kuramochi-Miyagawa et al., 2001) and the cnidarian *Podocoryne* (Seipel et al., 2004). The mammalian homologs were specifically expressed in the adult testis and were not detectable in other tissues, including the ovary. In adult zebrafish, *ziwi* was expressed exclusively in the gonads, and in earlier stages it was also present in tissue that undergoes active morphological rearrangements by proliferation and/or tissue that showed high proliferate capacity (Tan et al., 2002).

A *piwi* homolog was also found in *Platynereis dumerilii* (*Pdu-piwi*), in the 48hpf cDNA library located at EMBL, Heidelberg, with the internal accession number: 34DD03FM1, sequence analysis by Raible et al. (unpublished). The alignment of *Platynereis* piwi with other homologs is shown in chapter 6.1.6.

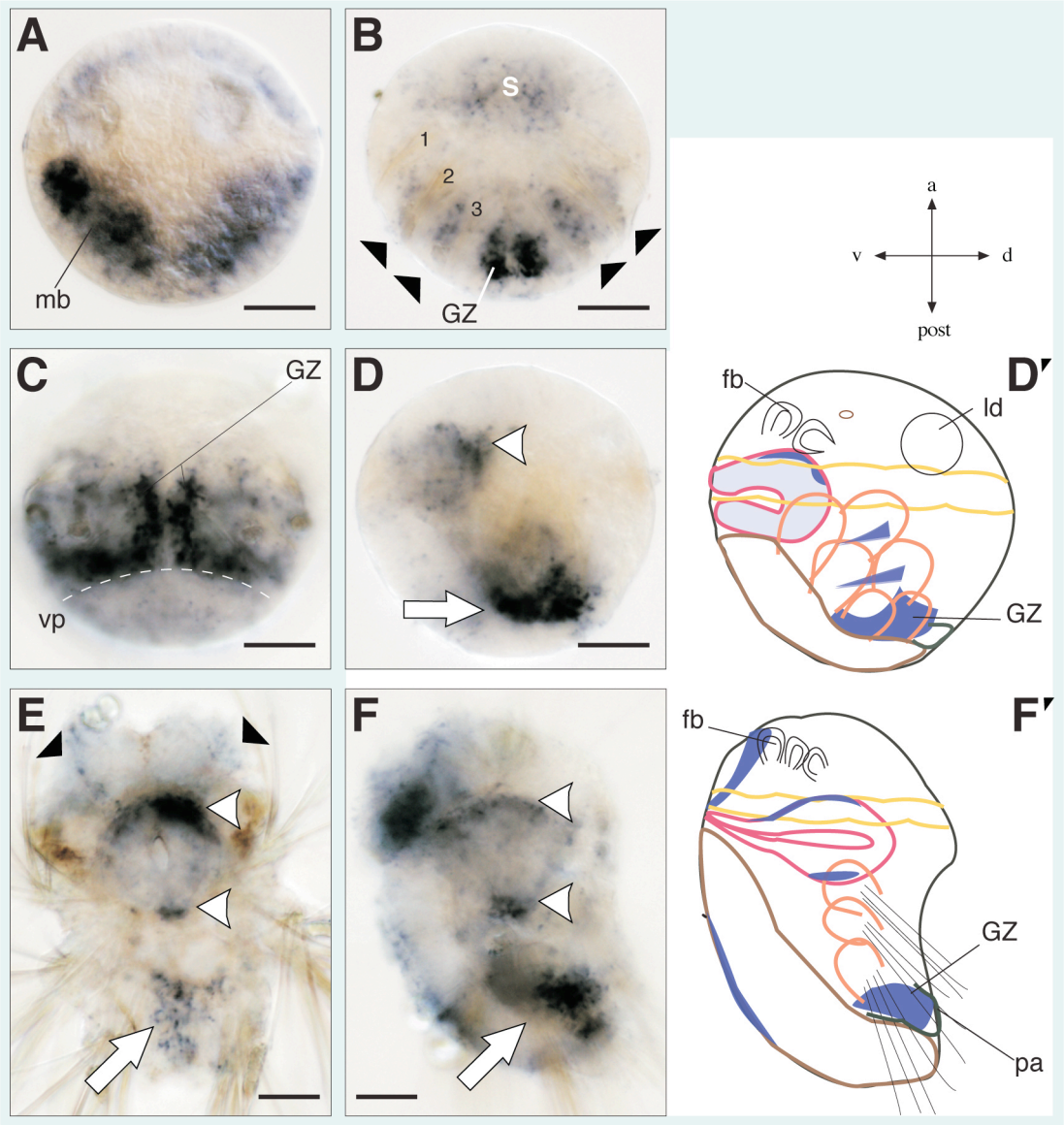


Figure 21. *Pdu-piwi* mRNA localization in *Platynereis dumerilii* embryos at 24 to 72hpf.

24hpf: (A) ventral view, strong *Pdu-piwi* staining in mesodermal bands and fainter staining in the brain region. 48hpf: (B) dorsal view, staining in the stomodaeum and at the posterior border of the 1st and 2nd segment, stronger mRNA localization in the posterior GZ. (C) posterior view, *Pdu-piwi* mRNA localization in the growth zone, as two parallel stripes of *Pdu-piwi* positive cells. The expression continues along the mesodermal bands on each side of the embryo, dorsally to the ventral plate. (D) lateral view, *Pdu-piwi* transcript in the stomodaeum accumulating in its most distal region and in the posterior GZ. 72hpf: (E) ventral view, *Pdu-piwi* mRNA in the most upper and lowest stomodaeal cells (mesodermal stomodaeal cells), in the antennal muscles of the brain, and in midline- cells. (F) lateral view, staining in the superficial cell of the ventral plate, strong staining in the posterior GZ and in most upper and in the lowest stomodaeal cells.

Colour usage: ventral plate (brown), stomodaeum (pink), pygidial area (green), chaetal sacs (light orange), prototroch (yellow) and *Pdu-piwi* mRNA (blue). *Abbreviations:* apical, a; posterior, post; hours post fertilization, hpf; lipid droplets, ld; frontal bodies, fb; pygidial area, pa; mesodermal bands, mb; stomodaeum, s; ventral plate, vp and growth zone, GZ. *Scale bar:* 50µm. Schematics of correspondent stages are represented with apostrophe (‘).

Pdu-piwi mRNA localization was studied 24hpf to 72hpf old larvae. It was co-localized with *Pdu-vas* and *Pdu-pl10*, although its expression was not completely similar. *Pdu-piwi* expression in the larvae was dotted, as shown in Figure 21, meaning that its mRNA was localized in the nucleus of each cell expressing it. At 24hpf the transcript was found in the mesodermal bands, and a weaker expression was noticed in the inner brain region (Figure 21A). At 36hpf, *Pdu-piwi* was strongly expressed in the growth zone, in the same pattern as *Pdu-vas* did. A weak "dotted" or nuclear staining was noticeable in the mesoderm between the chaetal sacs of the 1st and 2nd, and 2nd and 3rd segment (Figure 21B, black arrowheads), and in the stomodaeum. At 48hpf, the signal remained strong in the growth zone (Figure 21D, white arrow), superficially in two stripes on each side, posterior to the chaetal sacs of the first and of the second segment. In addition to this, *Pdu-piwi* was expressed in the stomodaeum, at higher levels in its distal region (Figure 21D, white arrowhead). A posterior view of 48hpf larvae showed strong *Pdu-piwi* staining in a double stripe oriented dorsal to ventral, at the position of the growth zone; thus, being co-expressed in this region with *Pdu-vas* and *Pdu-pl10*. Dorsal of the ventral plate, the mesodermal bands were expressing *Pdu-piwi* mRNA, as shown in Figure 21C. At 72hpf, *Pdu-piwi* positive cells were found in the mesodermal antennal muscles (Figure 21E, black arrowheads), in the mesodermal sheath of the stomodaeum (Figure 21E and F, white arrowheads), in the media, dorsal to the ventral plate, in the pygidial area, comprising the region of the growth zone (Figure 21E and F, white arrow), and in the midline, at the surface of the ventral plate (Figure 21F).

2.1.5 Nanos is involved in PGCs' formation

I cloned the *nanos* homolog in *Platynereis* since it is the candidate gene, based on available data on other homologs, because first, it was another candidate gene to investigate germline development in *Platynereis* and second, it would help to elucidate the link between germline development and axis formation.

In *Drosophila* both ***nanos*** and *piwi* are indispensable for germ stem cell maintenance (Cox et al., 1998; Lin and Spradling, 1997). *Nanos* was first identified in *Drosophila* and its RNA, like vasa protein, is a component of the germ plasm. This gene encodes an RNA binding zinc finger protein. *Nanos* mRNA is enriched in the posterior of the early *Drosophila* embryo, in the region where the germ plasm resides (Wang and Lehmann,

1991). *Nanos* in the fly is involved in proper germline development, since *nanos*-deficient PGCs do not develop normally (Forbes and Lehmann, 1998).

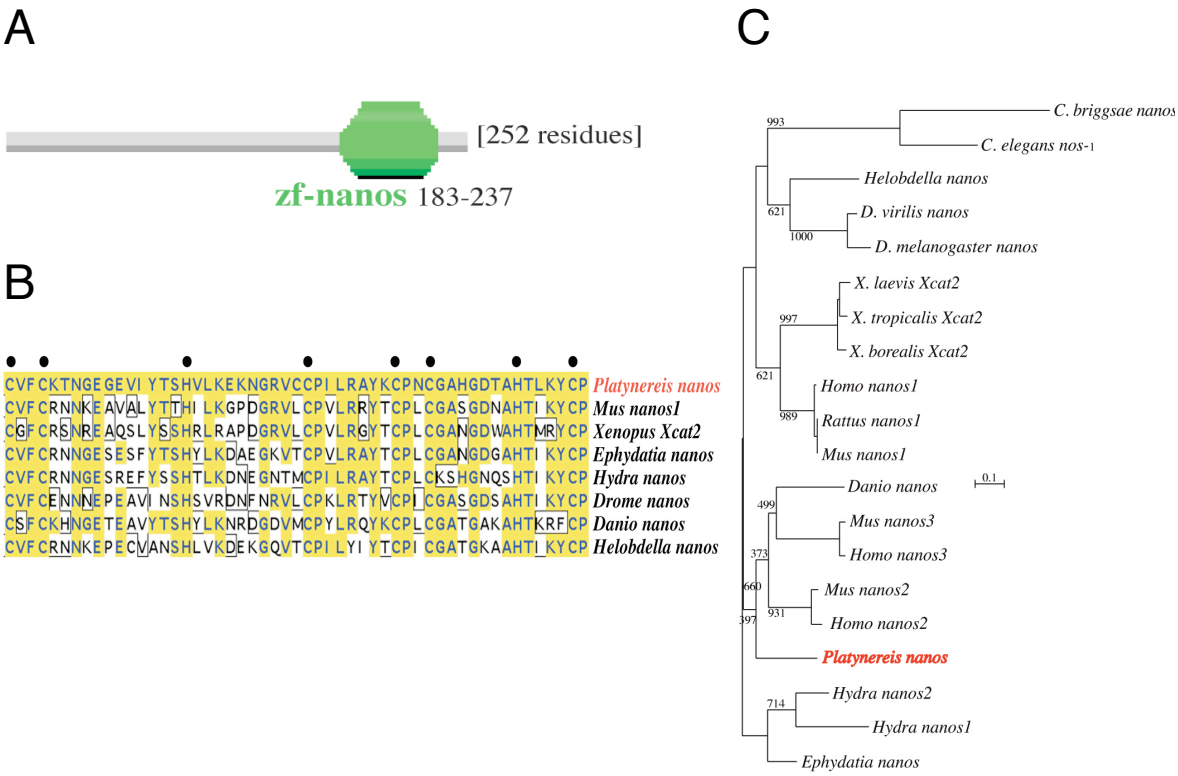


Figure 22. *Platynereis dumerilii nanos* (*Pdu-nanos*) gene encodes a zinc finger protein

(A) *Platynereis* nanos protein consists of 252 AA and contains a nanos type zinc finger RNA binding domain located towards the C-terminus (green). The position of the domain is depicted with numbers, on the left. The AA sequence was blasted against the protein families' database of alignments and HMMs (Pfam). (B) Comparison of the AA sequence of the zinc finger domain among nanos homologs from *Platynereis dumerilii* (*nanos*) *Mus musculus* (*nanos1*), *Xenopus tropicalis* (*Xcat2*), *Ephydatia fluviatilis* (*PoNos*), *Hydra magnipapillata* (*CNNOS1*), *Drosophila melanogaster* (*nanos*), *Danio rerio* (*Nos1*) and *Helobdella robusta* (*Hro-nos*). The characteristic C-terminal CCHC CCHC zinc finger domain with its conserved residues marked with a black point on the top. Amino acids that are conserved with *Platynereis* nanos are highlighted in yellow. (C) Phylogenetic analysis of nanos homologs using the Neighbour Joining method. *Platynereis* nanos (red) clusters closer to human- and mouse- nanos2 and nanos3 homologs than to homologs of other species.

Nanos mRNA codes the nanos zinc finger domain (Figure 22A and B), which is the only conserved region shared among the other homologs reported. Phylogenetic analysis made with the Neighbour Joining method show that *Platynereis* nanos is a true homolog for its family, with a slightly higher affinity to mouse- and human- *nanos2* and *nanos3* homologs (Figure 22C). There is no other nanos-related protein family, therefore I chose *Ephydatia*

fluviatilis (*PoNos*) together with the *Hydra* homologs as outgroup to construct this phylogenetic tree.

I performed WMISH in *Platynereis* early stages, larvae and adult worms. No *Pdu-nanos* expression was detected in 2-cell to 29-cell (~4.50hpf) stage. At 12hpf, *Pdu-nanos* was expressed in the hyposphere, two big cells in the posterior of the embryo were strongly stained (Figure 23A and B, white arrow), these cells could well be the mesodermal stem cells, also called primary mesoblasts, and were described by Wilson (1892). The primary mesoblasts were first noticeable in *Nereis* (*N. limbata* and *N. megalops*) at 14hpf. At 15hpf, the *Pdu-nanos* staining was observed in the mesodermal bands (Figure 23F and G-white arrow), and at high levels in the most posterior cells of the embryo (Figure 23G-white arrow).

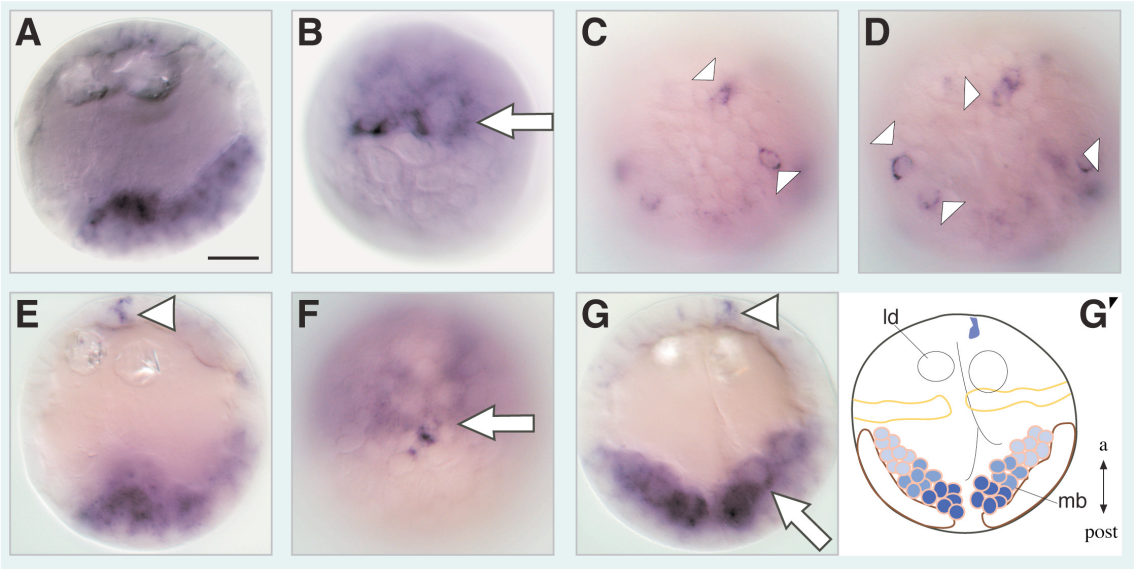


Figure 23. *Pdu-nanos* mRNA expression in 12hpf and 15hpf larvae

12hpf: (A) lateral view, *Pdu-nanos* expression in the hyposphere. (B) posterior view, Pdu-nanos mRNA localized in two big cells- white arrow. 15hpf: (C) superficial anterior view, and (D) deeper anterior view, 8 cells are expressing *Pdu-nanos* in the brain region-white arrowheads. (E) lateral view, *Pdu-nanos* mRNA in the hyposphere and in the brain, probably in the apo- white arrowhead. (F) posterior superficial view, *Pdu-nanos* transcript in the mesodermal bands, and detected at high levels in cells located in the centre and most posterior end of the embryo-white arrow. (G) ventral view, *Pdu-nanos* mRNA in mesodermal bands -white arrow, and in the apo -white arrowhead. (G') schematics of (G). Colour usage: ventral plate (brown), mesodermal bands (light pink), prototroch (yellow) and *Pdu-nanos* mRNA (blue). *Abbreviations:* apical organ, apo; mesodermal bands; mb; lipid droplet, ld; apical, a and posterior, post. *Scale bar:* 50µm for all pictures.

At 48hpf, *Pdu-nanos* mRNA was found in some cells of the growth zone (Figure 24D-white arrow). At 4dpf, four to five *Pdu-nanos* positive cells were found in the growth zone. **These cells are most likely primordial germ cells** (Figure 24H, and depicted in schematics in Figure 24H'-green), since these were most probably the cells that at this stage were synthesizing vasa protein. In worms older than 6dpf, no *Pdu-nanos* signal was detected in the growth zone by WHMISH.

I then performed a semi-quantitative RT-PCR, in order to corroborate the WHMISH results. *Pdu-nanos* was first detected at 5hpf and it was still present at high levels at 5dpf. Juveniles and worms in epitoky did not express *Pdu-nanos* mRNA (Figure 25). Neither females nor unfertilized oocytes expressed *Pdu-nanos* mRNA. *Pdu-nanos* was first detected after the midblastula transition, which is completed after the 5^{th} cleavage stage at 4.50hpf (Dorresteijn, A., personal communication).

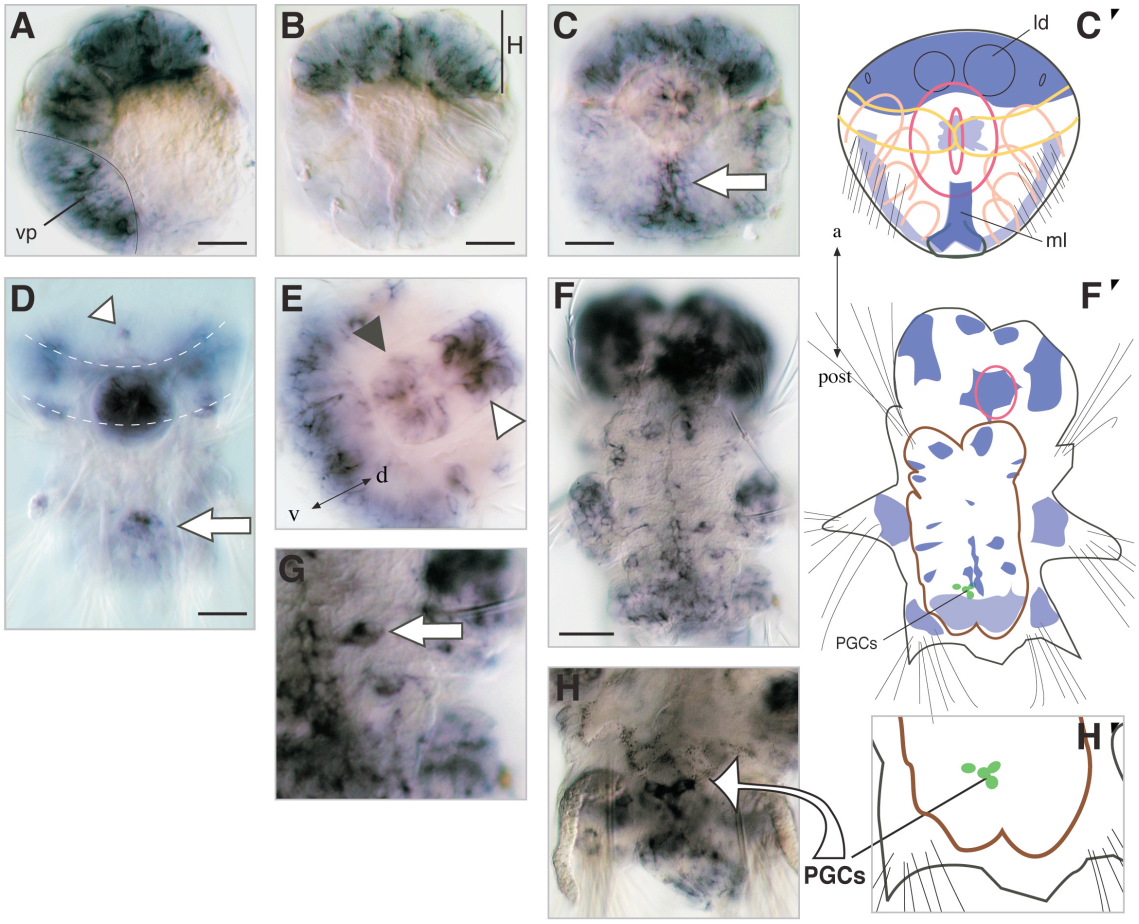


Figure 24. *Pdu-nanos* mRNA localization in 48hpf to 4dpf old worm

48hpf: (A) lateral view, *Pdu-nanos* mRNA localization in the brain region, stomodaeum and ventral plate. (B) dorsal view, *Pdu-nanos* transcript in the complete head region. (C) ventral view, *Pdu-nanos* mRNA expression in the brain, stomodaeum and in a vertical stripe demarcating the midline- white arrow. 72hpf: (D) ventral view, *Pdu-nanos* mRNA localization in the apo-white arrowhead, and two parallel stripes in the brain. *Pdu-nanos* positive cells found in the GZ- white arrow. (E) apical view of the stomodaeum, the upper *Pdu-nanos* positive brain stripe is a half circle to the ventral side. Two stomodaeal regions were expressing *Pdu-nanos* mRNA, the most distal part expressing the transcript at high levels, and the most ventral region showing a much weaker staining. 4dpf: (F) ventral superficial view, *Pdu-nanos* mRNA detected in the brain, stomodaeum. The transcript is expressed in some peripheral-, midline- and parapodial neurons. (G) is enlarged from (F), neurons expressing *Pdu-nanos*- white arrow. (H) ventral view of the posterior of the worm, *Pdu-nanos* is expressed in PGCs-white arrow, which are located above the pygidium and posterior to the forming gut.

Colour usage: ventral plate (brown), stomodaeum (purple), chaetal sacs (light orange), prototroch (yellow), pygidial area (green), PGCs (light green) and *Pdu-nanos* (blue and light green). *Abbreviations:* primordial germ cell(s), PGC(s); midline, ml; ventral plate; vp and head, H. *Scale bar:* 50µm, except for (E), (G) and (H). Schematics of correspondent stages are represented with apostrophe (‘).

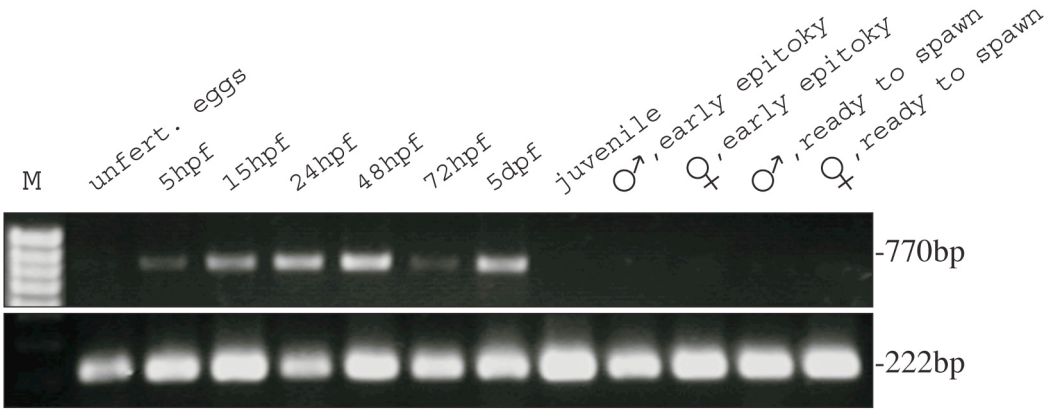


Figure 25. RT-PCR results for *Pdu-nanos*

Pdu-nanos transcript (770bp fragment) is first detected at 5hpf. It remains present until 5dpf, with a significant drop at 72hpf. A control with *Pdu-actin* is included in the picture below (222bp fragment). "M" stands for the DNA ladder marker. The primers used were *Pdnos spec fw xba1* and *pdnos spec rev hindIII*.

2.2 Axis formation

2.2.1 A/P patterning

The genes that play a role in germline development are often involved in the process of axis formation. Investigating these genes in *Platynereis*, being an archetypical member of the lophotrochozoans, might elucidate whether this link is also valid for this phylogenetic group, thus enlightening the evolutionary conservation of this link among bilaterians. For this reason, I have cloned and investigated few genes, which are tightly linked in both mechanisms.

2.2.1.1 *Nanos* - a putative *hunchback* repressor- is expressed in the nervous system in *Platynereis*

In *Drosophila*, *nanos* is translated in the posterior region of the early embryo, generating a protein gradient with higher levels to the posterior (Gavis and Lehmann, 1994). Nanos, together with pumilio protein, binds to the 3' UTR of *hunchback* mRNA repressing its translation, and therefore creating a *hunchback* gradient, which in turn regulates other segmentation genes specifying regional identity along the A/P axis (Murata and Wharton, 1995; Wharton et al., 1998; Wharton and Struhl, 1991).

In *Platynereis*, eight cells expressing *Pdu-nanos* were detected at 15hpf in the brain (Figure 23C and D, white arrowheads). At 48hpf, there was a strong *Pdu-nanos* mRNA expression in the entire brain, stomodaeum and ventral plate (Figure 24A -C). A ventral

view of this stage showed strong *Pdu-nanos* staining in the midline (Figure 24C-white arrow). At 72hpf, there were *Pdu-nanos* positive cells in the apical organ (Figure 24D-white arrowhead). The expression was found in two superficial ventral stripes on the head, the first one overlapped the *Pdu-pax6* region (Arendt et al., 2002) and the second stripe was at the boundary of the 0 and 1st segment. *Pdu-nanos* mRNA was found in the stomodaeum (Figure 24D). The staining in the stomodaeum was divided in two regions, with the distal most region expressing high levels of *Pdu-nanos* (Figure 24E-white arrowhead), and the most ventral region of the stomodaeum showing slightly lower levels of *Pdu-nanos* expression (Figure 24E-grey arrowhead). At 4dpf, *Pdu-nanos* mRNA was detected in some peripheral-, midline- and parapodial neurons (Figure 24F and G-white arrow).

The strong staining in the brain and stomodaeal cells persisted at 4dpf, and was similar to the expression pattern described for worms at 72hpf. By 6dpf the only *Pdu-nanos* signal detected was faint and fuzzy in the region of the brain. At 23dpf the expression in the brain was nearly undetectable. In worms older than 23dpf, no *Pdu-nanos* signal was detected by WHMISH.

To answer the question whether *Platynereis* nanos protein plays a role in translational repression of *hunchback* and in the establishment of the A/P axis across Bilateria, I had to compare the localization of *Platynereis* nanos protein with the territory of *hunchback* expression. For a preliminary test, we used antibodies against the leech nanos protein (Pilon and Weisblat, 1997). The antibody was a kind gift from David Weisblat. Unfortunately, these antibodies did not cross-react with *Platynereis* nanos protein. Therefore, we started to raise antibodies against *Platynereis* nanos (Schwager, 2004).

2.2.1.2 *Hunchback* expression in *Platynereis* suggests a role in proper segment formation

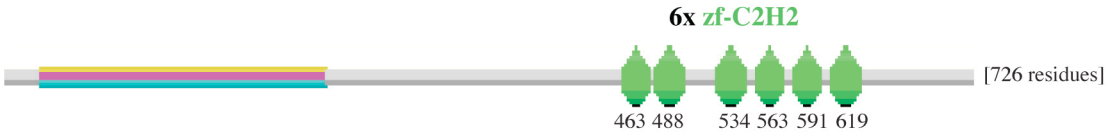
In *Drosophila hunchback* is classified as a gap gene that has a pivotal role in the antero-posterior (A/P) pattern formation. It was first characterized in *Drosophila* as a zinc-finger transcription factor (Hulskamp et al., 1990; Lehmann and Nusslein-Volhard, 1987). Hunchback protein gradient along the A/P axis establishes the thoracic and abdominal segments of the *Drosophila* embryo (Struhl et al., 1992). This gradient is generated at the blastoderm stage; bicoid protein activates *hunchback* transcription at the anterior pole (Simpson-Brose et al., 1994) and nanos and pumilio proteins repress its translation at the

posterior pole. The studies in *Drosophila* demonstrated that translational repression of *hunchback* depends on the 3' UTR of the *hunchback* transcript, which contains the *nanos* response element, the NRE, which is recognised by pumilio protein, which in turn recruits nanos protein (Murata and Wharton, 1995; Wharton et al., 1998; Wharton and Struhl, 1991). Hunchback protein gradient, in turn, regulates other segmentation genes and the *hox* genes, which are known to specify regional identity along the A/P axis.

A fragment of *Platynereis hunchback* (*Pdu-hb*) was already cloned (50AA long) and the accession number is AAA29792. I raced the gene towards both sides, and obtained the 5' end and part of the 3' end. The sequence of the gene was completed by the data obtained from the *Platynereis* internal database of 48hpf cDNA library (EMBL-Heidelberg).

The *hunchback* homolog for *Platynereis dumerilii* (*Pdu-hb*) is 2418bp long, its ORF starts at position 67 and it codes a 726 AA protein, which contains a total of 6 C2H2 type zinc fingers (Figure 26A), five of them are also present in homolog of other species (Figure 27A). The C2H2 zinc finger is the classical zinc finger domain and is known to bind to DNA. The 3' UTR of *Pdu-hb* comprises a putative NRE (Figure 26B). The phylogenetic tree in Figure 27B shows *Platynereis* hunchback as a true homolog of its family, and it is more likely to cluster with the leech hb (Lzf2). There is no *hunchback* homolog known in vertebrates; homologs have been reported in insects and in some worms. I used two members of the rest protein family as outgroup to construct this tree, since members of this family contain 5 repetitive C2H2 zinc fingers, and it is the family of proteins that shows the most similarity to hunchback homologs. Rest stands for RE1-silencing transcription factor, and mouse rest is required in vivo for repression of multiple neuronal target genes during embryogenesis (Chen et al., 1998).

A



B

Figure 26. *Platynereis* hunchback protein domain architecture and alignment of *hunchback* putative NREs from different species

(A) *Platynereis* hunchback contains 6 zinc fingers (green), belonging to the C2H2 type, which is the classical zinc finger domain. The position of each zinc finger is illustrated with numbers, below. The AA sequence was blasted against the protein families' database of alignments and HMMs (Pfam). (B) Alignment of the *hunchback* NREs from insects and *Platynereis* (red). Species used are *Drosophila virilis*, *Drosophila melanogaster*, *Locusta migratoria*, *Musca domestica*, *Schistocerca americana*, *Tribolium castaneum* and *Platynereis dumerilii*.

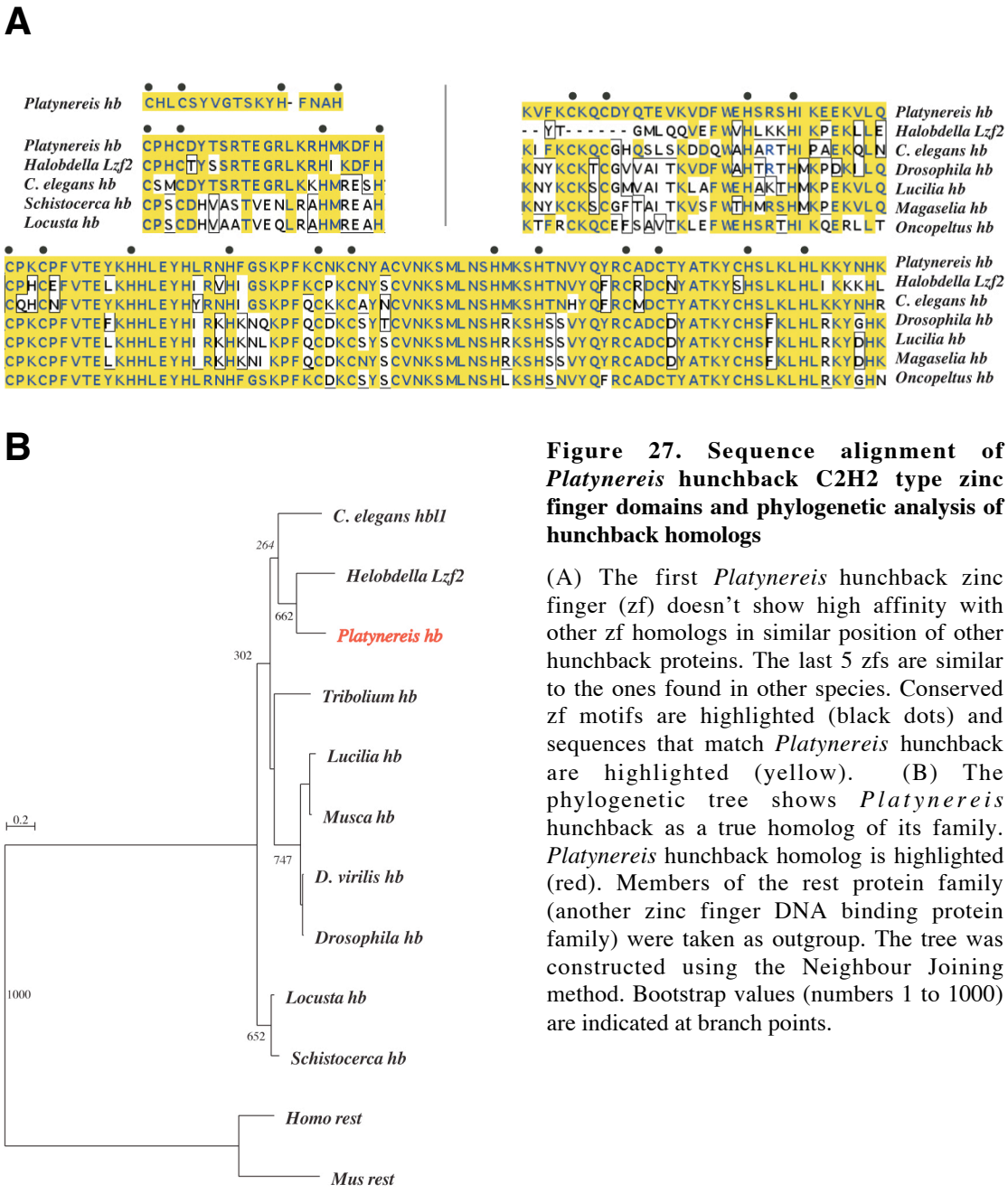


Figure 27. Sequence alignment of *Platynereis* hunchback C2H2 type zinc finger domains and phylogenetic analysis of hunchback homologs

(A) The first *Platynereis* hunchback zinc finger (zf) doesn't show high affinity with other zf homologs in similar position of other hunchback proteins. The last 5 zfs are similar to the ones found in other species. Conserved zf motifs are highlighted (black dots) and sequences that match *Platynereis* hunchback are highlighted (yellow). (B) The phylogenetic tree shows *Platynereis* hunchback as a true homolog of its family. *Platynereis* hunchback homolog is highlighted (red). Members of the rest protein family (another zinc finger DNA binding protein family) were taken as outgroup. The tree was constructed using the Neighbour Joining method. Bootstrap values (numbers 1 to 1000) are indicated at branch points.

I performed WHISH with *Pdu-hb* dig-labelled probes in most of the developmental stages of this polychaete (starting from 2-cell stage to fully mature worms, although the staining in mature worms was still problematic (see chapter 2.1.1). There was no staining in embryos of 2- to 29-cell stage. *Pdu-hb* mRNA was first detectable at 15hpf, in the centre of the brain (Figure 28A-white arrow), dorsal to the apical organ (Figure 28C-white arrowhead). It was detected in the hyposphere, in the mesodermal bands, and was excluded from the stomodaeal precursor cells and the pygidial area (Figure 28A-C). At 19hpf the

staining in the brain corresponded to the larval eye region on each side of the embryo (Figure 28E, F –white arrowheads, and H). *Pdu-hb* expression in the hyposphere persisted in the mesodermal bands (Figure 28D-G).

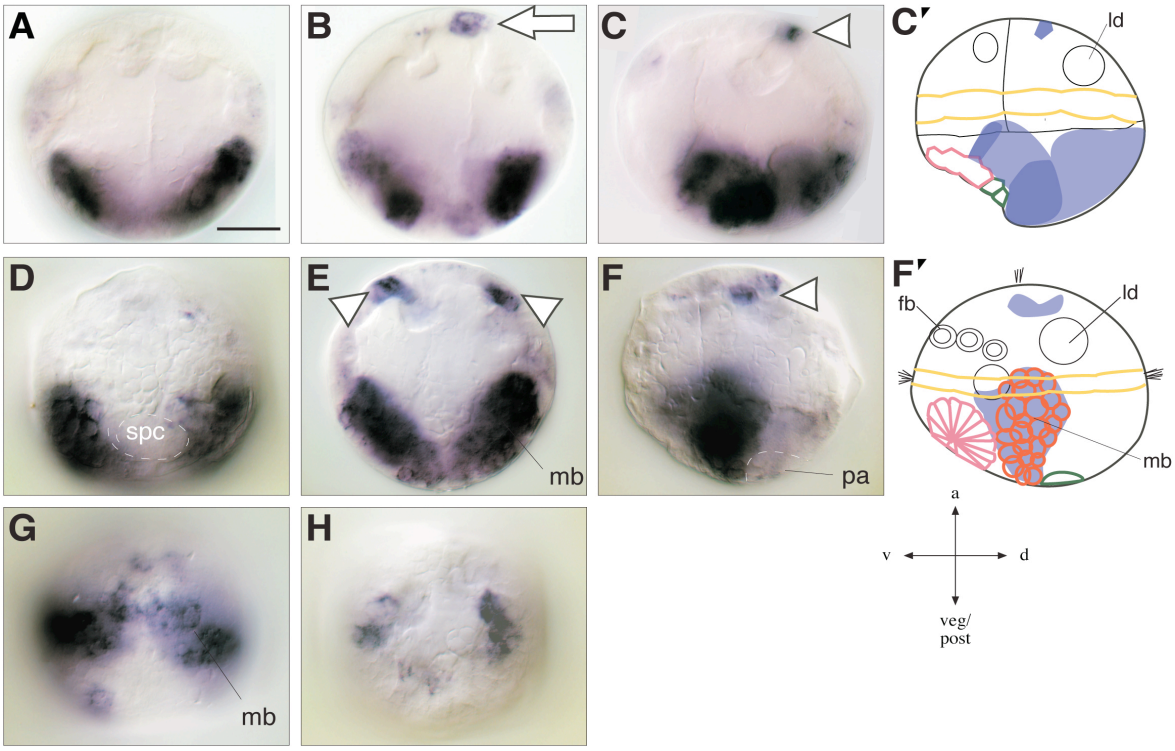


Figure 28. *Pdu-hunchback* mRNA localization embryos of 15 and 19hpf

15hpf: (A) ventral view, staining in the hyposphere, in the mb. The staining reaches the most outer layer of the hyposphere. (B) dorsal view, *Pdu-hb* transcript in cells of the centre of the brain and in the hyposphere, in the mesodermal bands [mb]. (C) lateral view, *Pdu-hb* transcript in the hyposphere, excluding the precursors of the stomodaeal precursor cells (spc) and the pygidial area (pa). Brain cells located dorsal to the APO express *Pdu-hb* mRNA. (D) superficial ventral view, *Pdu-hb* mRNA in the hyposphere, in the mb and excluding spc. (E) dorsal view, *Pdu-hb* mRNA in mb and in brain cells on each side of the embryo-white arrowheads. (F) lateral view, *Pdu-hb* mRNA in mb and in the brain. (G) superficial posterior view, *Pdu-hb* mRNA in mesodermal bands reaching in the centre of the embryo the most posterior cells. (H) superficial anterior view, *Pdu-hb* transcript in two half moon shaped region, the larval eye region.

Colour usage: ventral plate (brown), spc and stomodaeum (pink), pygidial area (green), mesodermal bands (orange), chaeta sacks (light orange), prototroch (yellow) and *Pdu-hb* mRNA (different shades of blue). *Abbreviations:* frontal bodies, fb; lipid droplets, ld; mesodermal bands, mb; stomodaeal precursor cells, spc; pygidial area, pa; apical, a; ventral, v; dorsal, d; vegetal, veg and posterior, post. *Scale bar:* 50µm, for all pictures in this figure. Schematics of correspondent stages are represented with apostrophe (‘).

At 22hpf, *Pdu-hb* mRNA was localized in the brain, in two half moon like structures (Figure 29B, schematics on the left). The transcript was found in the inner region of the forming chaetal sacs (Figure 29B-green square, enlarged in C). At this stage, four *Pdu-hb* positive cells were detected - two on each side of the ventral part of the embryo (Figure

29A-white arrowheads). At 24hpf these cells were localized closer to each other in the region where the stomodaeum is forming (Figure 29D-white arrowheads). These cells could well be the stomatoblasts, which are the cells that give rise to the inner mesodermal layer of the stomodaeum. There was *Pdu-hb* staining in the mesodermal bands and in inner region of the brain (Figure 29E). Viewed the embryo from anterior (Figure 29H), the staining was almost identical to what could be seen from the posterior view (Figure 29I). The staining was exclusive in the ventral side of the embryo, with the exception of two groups of *Pdu-hb* positive cells one on each side of the embryo, located dorsally (Figure 29G-white arrowhead). At 30hpf, no *Pdu-hb* expression was detected in the brain anymore. The expression resided at the posterior border of the first segment and stronger expression was visible at the posterior border of the 2nd and 3rd segment and in the pygidium (Figure 30A and B). At 48hpf, *Pdu-hb* transcript was expressed in the mesodermal bands, and at higher levels in the whole 2nd and 3rd segment, with an expression gap between these segments (Figure 30D and E). I cloned and investigated *Pdu-troponinI*, which is a marker for muscles, to compare it with the expression of *Pdu-hb* (as well as other genes expressed in the mesodermal linage). In muscles, *troponinI* is an important element in the protein complex, which regulates sliding of thin over thick filaments (Farah and Reinach, 1995; Lehrer and Geeves, 1998; Maytum et al., 2003). *Pdu-troponinI* mRNA localization at 48hpf overlapped with *Pdu-hb* expression in the mesodermal bands, although *Pdu-hb* went farther in all the layers of the 2nd and 3rd segment including the pygidium, which lacked *Pdu-troponinI* expression at this stage (Figure 30C). At 72hpf, *Pdu-hb* expression was restricted to the ectoderm epithelia (Figure 30G-white arrow and H). A comparison with the *Pdu-troponinI* the staining demonstrated, that *Pdu-hb* staining was excluded from *Pdu-troponinI* expression region (Figure 30J and I). At 85hpf, *Pdu-hb* mRNA persisted in the ectoderm epidermis.

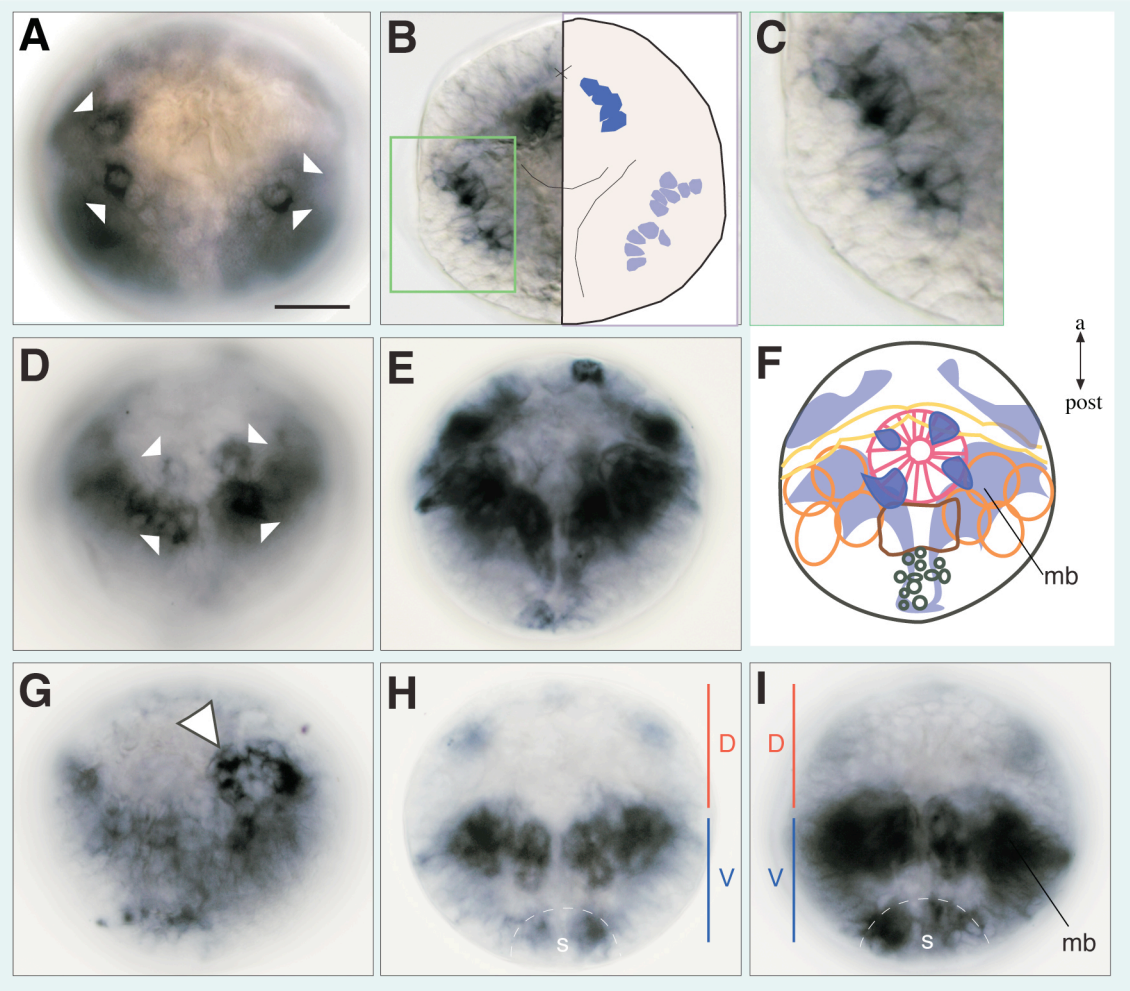


Figure 29. *Pdu-hunchback* mRNA expression in embryos of 22 and 24hpf

22hpf: (A) superficial ventral view, *Pdu-hb* mRNA localization in 4 cells around the forming stomodaeum (see white arrows). (B) ventral view, *Pdu-hb* transcript is localized as two half crescent like structures in the centre of the brain and in cells from the inner region of the forming chaetal sacs, enlarged in (C). 24hpf: (D) superficial ventral view, 4 stomatoblasts and surrounding cells express *Pdu-hb* mRNA. (E) ventral view, *Pdu-hb* mRNA expression in mesodermal bands that extend to the few cells at the tip of the pygidial area and in similar bands on the ventral side of the brain. (F) ventral view, schematic representation of *Pdu-hb* mRNA localization. (G) superficial dorso-lateral view, expression of *Pdu-hb* mRNA in cells located dorso-lateral. (H) anterior view (deep), *Pdu-hb* mRNA expression in bands of the ventral region of the brain, and in stomodaeal cells. (I) posterior view, *Pdu-hb* mRNA expression in mesodermal bands and in stomodaeal cells.

Colour usage: ventral plate (brown), spc and stomodaeum (pink), pygidial area (green), chaetal sacs (light orange), prototroch (yellow) and *Pdu-hb* mRNA (different shades of blue). *Abbreviations:* apical, a; posterior, post, mesodermal bands, mb; stomodaeum, s; dorsal, D and ventral, V. *Scale bar:* 50µm, for all pictures in this figure.

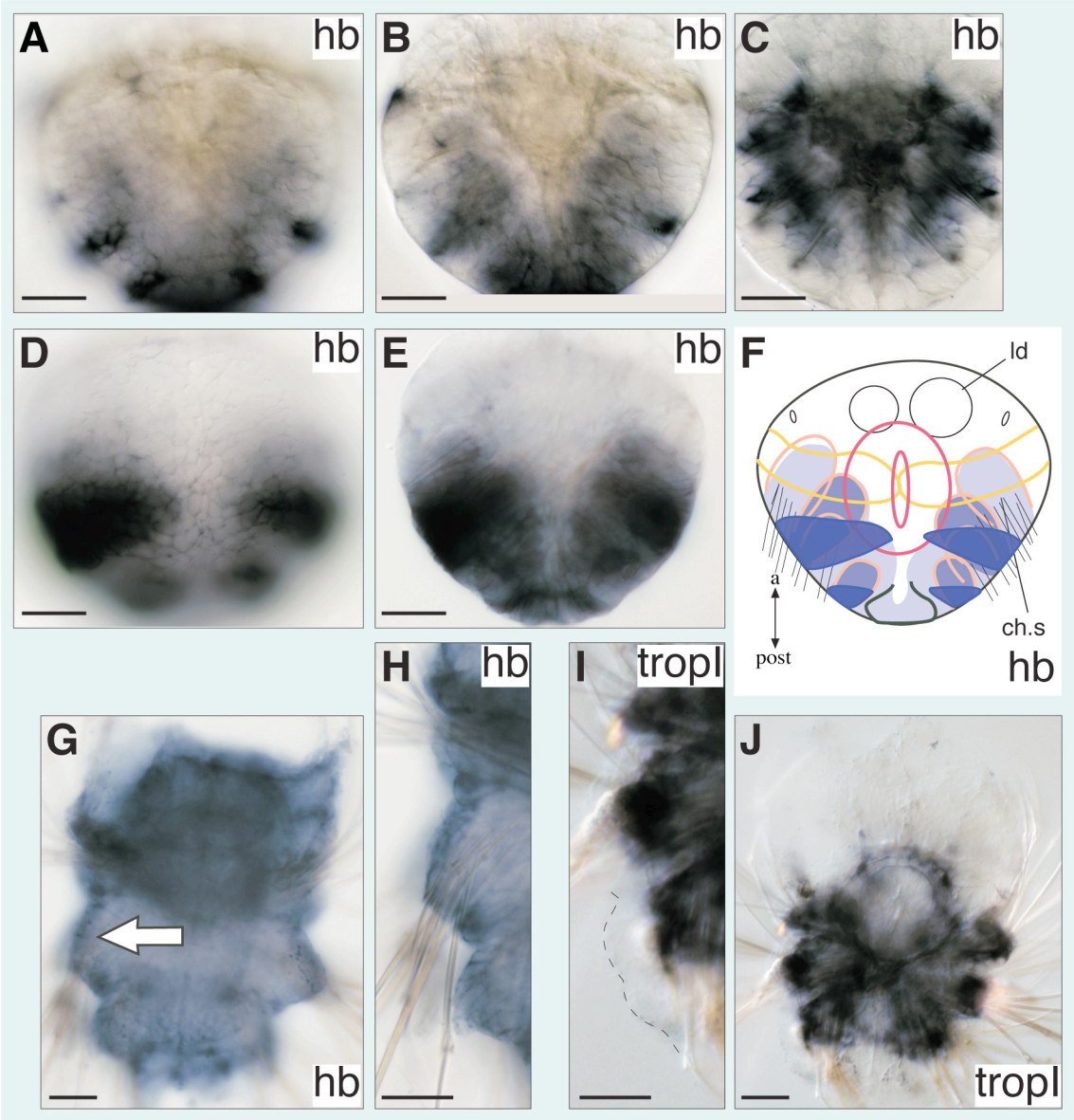


Figure 30. *Pdu-hb* and *Pdu-troponinI* mRNA localization in larvae of 30, 48 and 72hpf.

30hpf: (A) superficial ventral view, faint *Pdu-hb* staining at the posterior boundary of the first segment, stronger staining at the posterior boundary of the 2nd and 3rd segment and in the pygidium. (B) dorsal view, faint *Pdu-hb* in mesodermal bands, stronger expression in the posterior region of the 2nd, 3rd segment and in the pygidium. 48hpf: (C) dorsal view, *Pdu-troponinI* mRNA in muscle cells. (D) superficial ventral view, strong *Pdu-hb* mRNA localization in the 2nd and 3rd segment. (E) dorsal view, *Pdu-hb* expression in all three segments and in the pygidium. A noticeable stronger expression is observed in the 2nd segment and in the posterior boundary of the 3rd segment. (F) ventral view, schematics of *Pdu-hb* mRNA localization. 72hpf: (G) ventral view, *Pdu-hb* transcript observed along the ectoderm epithelia, enlarged in (H). (J) ventral view, *Pdu-troponinI* mRNA expression in muscles cells, enlarged in (I).

Colour usage: stomodaeum (pink), pygidium (green), chaetal sacs (light orange), prototroch (yellow) and *Pdu-hb* mRNA (different shades of blue). *Abbreviations:* apical, a; posterior, post; lipid droplet, ld and chaetal sacs, ch.s. *Scale bar:* 50µm.

RT- PCR experiments confirmed these results and added new information on *Pdu-hb* expression throughout *Platynereis* development (Figure 31). *Pdu-hb* transcript was first transcribed at 15hpf and remained active in the following developmental stages except for females in early epitoky, where no transcript was detected. In females that were ready to spawn the transcript was present although at noticeably lower levels when compared to males that were ready to spawn. Since the transcript is not present in unfertilized eggs, or in 5hpf larvae, any maternal contribution of the transcript in the embryo is excluded.

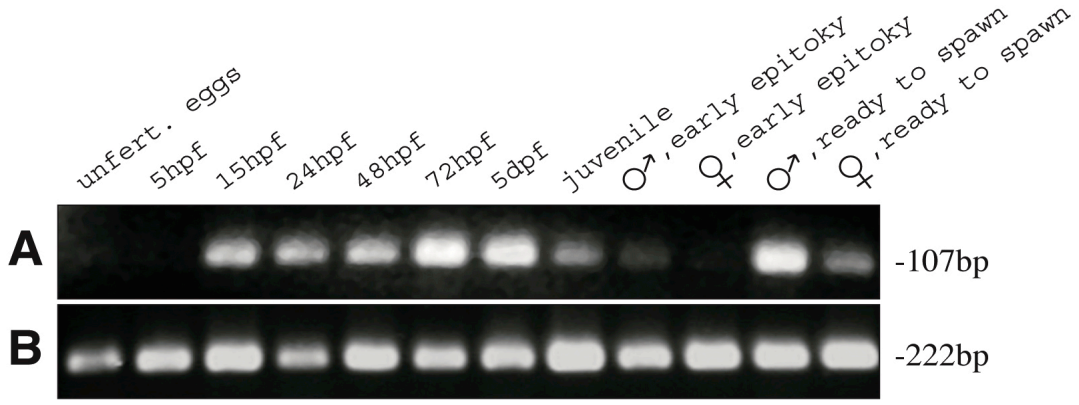


Figure 31. RT-PCR from *Platynereis hunchback*

(A) *Pdu-hb* transcript (107bp fragment) is first detected at 15hpf. The transcript is active in the following stages of development excluding males that are early in epitoky, where no *Pdu-hb* mRNA is detected. The levels of expression in males that are ready to spawn are noticeable higher when compared to females of the same developmental stage. (B) Control with *Pdu-actin* (222bp fragment). The primers used were *HbspecUp2* and *HbspecLo2* (see chapter 4.11.3).

To answer the question whether nanos represses *hunchback* translation in *Platynereis*, it was necessary to investigate *Platynereis* hunchback protein (as well as *Platynereis* nanos protein, discussed in chapter 2.1.5). For this reason, I performed immunohistochemistry in *Platynereis dumerilii* larvae and western blot with the polyclonal antibody raised against the hunchback protein of the leech (Iwasa et al., 2000). The antibodies were a kind gift from Rob Savage. Leeches belong to the lophotrochozoans, as does *Platynereis*, therefore it was likely that these antibodies also would recognize the *Platynereis* hunchback protein. Indeed, these antibodies (1:1000 dilution) recognised a band (among two other, see Figure 32-black arrows) at 84kDa in *Platynereis dumerilii* protein extract of a single batch of 24hpf (Figure 32-red arrow). This band might correspond to *Platynereis* hunchback protein, since its theoretical molecular weight was estimated to be around 82.4kDa. Immunocytochemistry was performed with the leech hunchback antibody on different developmental stages (8hpf to 72hpf), with 1:30 dilution, but unfortunately no positive

signal was detected when compared to the background of the negative control for each stage.

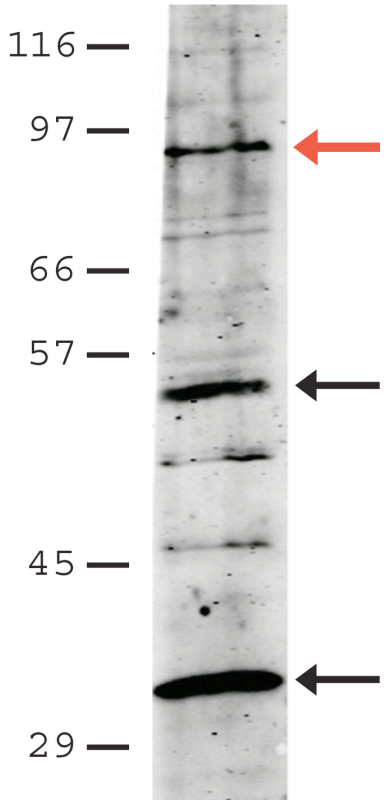


Figure 32. Western blot with polyclonal antibodies against the leech hunchback protein

Platynereis protein extract of one 24hpf batch. 50µg of protein extract per lane loaded. The dilution used was 1:1000. These antibodies cross-react with several bands (arrows), from which the one highlighted (red arrow) corresponds to 84kDa and is likely to correspond to *Platynereis* hunchback protein, since the theoretical molecular weight of the *Platynereis* hunchback protein is estimated to be 82.4kDa, according to sequence obtained form the cDNA. Molecular weight Marker is represented in kDa, numbers on the left.

2.2.1.3 *Par-1* and *pumilio* homologs exist for the polychaete

Par-1 was first characterized for the nematode *C. elegans*. It codes for a serine/threonine kinase. Transcripts localized to the posterior of the zygote and it was required for antero-posterior axis formation. Mutants for *par-1* in the nematode caused a symmetric first division and blocked the segregation of the P granules and other determinants along the A/P axis (Guo and Kemphues, 1995; Kemphues et al., 1988). The *Drosophila par-1* mutant failed to localize *oskar* mRNA to the posterior pole of the oocyte, resulting in embryonic patterning defects. The posterior localization of *oskar* mRNA was the key step for the assembly of polar granules, because oskar protein recruited components of the polar granules such as vasa protein (Ephrussi et al., 1991; Ephrussi and Lehmann, 1992; Kim-Ha et al., 1991).

I cloned a 738bp long fragment of *par-1* homolog in *Platynereis dumerilii* (*Pdu-par-1*). The last 200AA of this fragment comprised the par-1 kinase domain (Figure 33A and B).

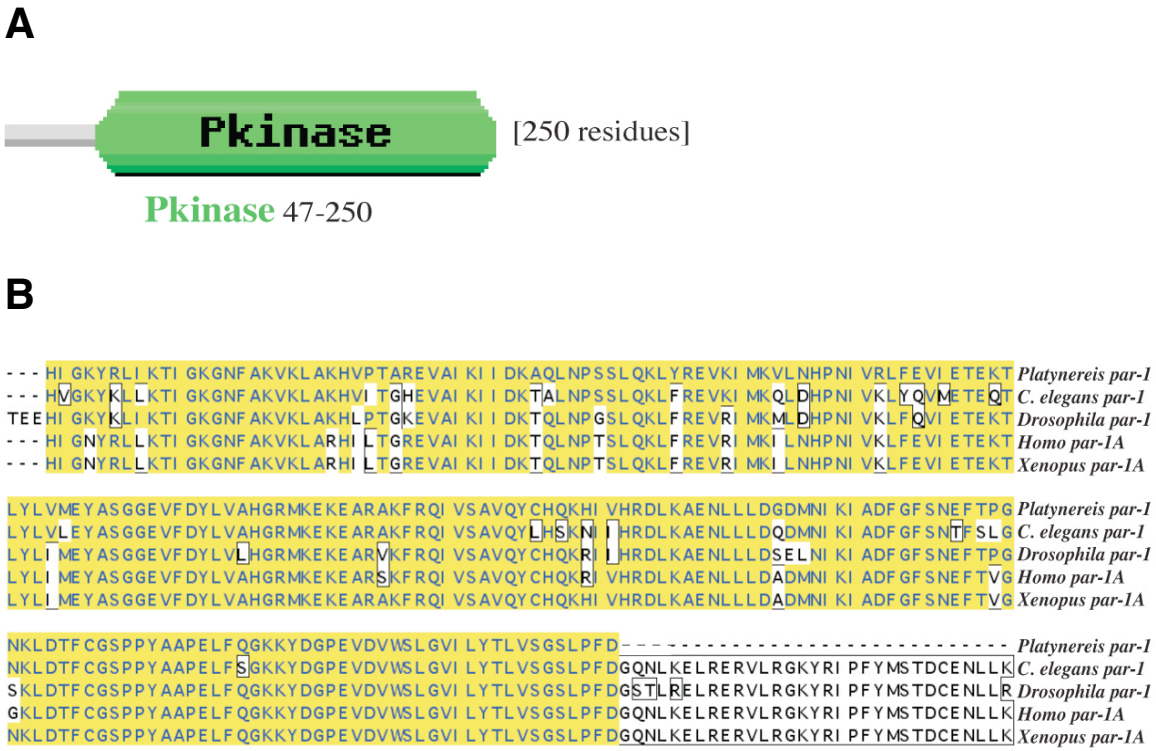


Figure 33. *Platynereis dumerilii* par-1 protein domain architecture and sequence alignment of its kinase domain.

(A) The fragment of *Pdu-Par-1* codes 250 AA, where the last 200AA comprises part of the protein kinase domain. The position of the domain is depicted with numbers, on the right. The AA sequence was blasted against the protein families' database of alignments and HMMs (Pfam). (B) Comparison of the AA sequence of the kinase domain among par-1 homologs for *Platynereis dumerilii*, *C. elegans*, *Drosophila melanogaster*, *Homo sapiens*, and *Xenopus laevis*. Amino acids that are conserved with respect to *Platynereis* par-1 protein are highlighted (yellow).

I investigated *Pdu-par-1* expression pattern in 24hpf to 5dpf old larvae. The gene was broadly expressed at 15hpf. At 24hpf *Pdu-par-1* mRNA was ubiquitous distributed throughout the whole embryo, except for the two macromeres and the region where the growth zone formed (Figure 34B- white arrow). At 5dpf, *Pdu-par-1* mRNA localization was restricted to the area comprising the brain, in particularly the transcript was found in the mid-dorsal and central brain, where the staining was much stronger (Figure 34C-white arrow and D).

RT-PCR results demonstrated that *Pdu-par-1* transcripts were present at all stages of development, with females ready to spawn, unfertilized eggs and worms at 5dpf showing higher levels expression (Figure 35).

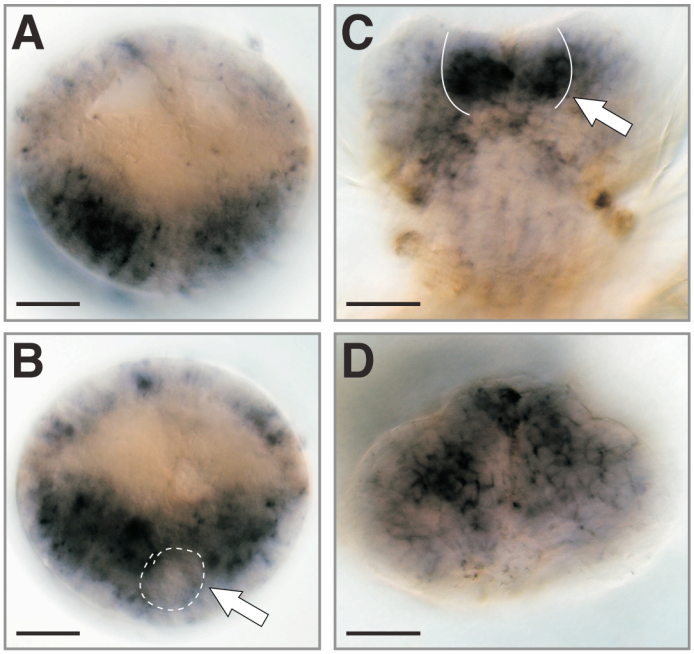


Figure 34. *Pdu-par-1* mRNA expression

24hpf: (A) ventral view, *Pdu-par-1* mRNA is expressed in the hyposphere. Low levels of expression are detected in the posterior end of the embryo, and in the brain. There is no *Pdu-par-1* expression in the macromeres. (B) dorsal view, *Pdu-par-1* transcript detected in the brain and in whole hyposphere, excluding a small gap at the posterior of the embryo, at the position of the posterior growth zone-encircled in white, white arrow. 5dpf: (C) ventral view of the head, *Pdu-par-1* is expressed in the brain, at higher levels in the centre of the brain-white arrow. (D) apical view, *Pdu-par-1* mRNA is expressed in the brain, with higher levels in the mid-dorsal region. *Scale bar:* 50μm.

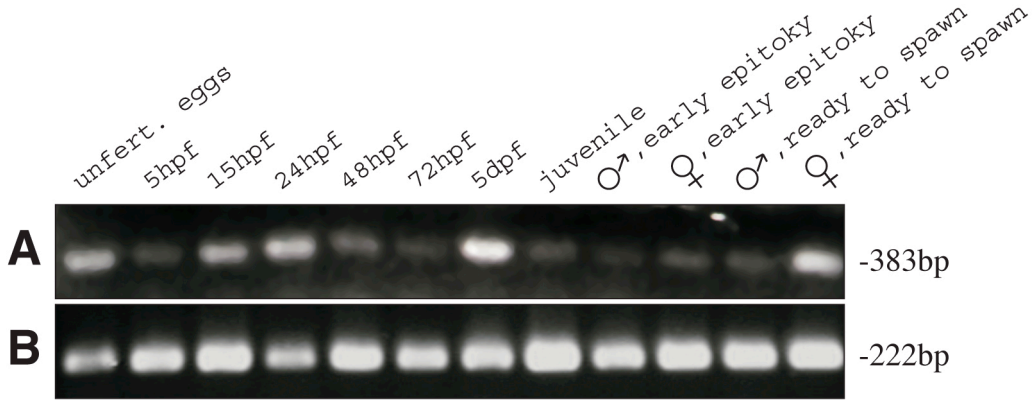


Figure 35. RT-PCR from *Pdu- par-1*

(A) *Pdu-par-1* transcript is detected in all developmental stages. High levels of expression are noticeable in females that are ready to spawn, unfertilized eggs and in worms at 5dpf. (B) Control with *Pdu-actin* (222bp fragment). The primers used were *Par-1_spec_U1* and *Par-1_spec_L1* (for sequence see chapter 4.11.3).

Pumilio is an RNA binding protein that belongs to the Puf protein family. The name "Puf" derives from pumilio protein in *Drosophila melanogaster* and *fem-3* binding factor (FBF) protein in *C. elegans* (Zamore et al., 1997; Zhang et al., 1997). Pumilio has been best characterized in *Drosophila*. During embryogenesis, pumilio protein binds to nanos-responsive elements (NREs) in the maternal *hunchback* mRNA. Translational repression of *hunchback* mRNA by pumilio protein, which is at the same time anchored to the posterior

by nanos protein, produces an antero-posterior gradient of hunchback protein that is essential for abdomen formation (Sonoda and Wharton, 1999; Wharton et al., 1998). Pumilio protein (together with nanos protein) is also required to regulate embryonic and post-embryonic primordial germ cell development, gonadogenesis and oogenesis (Asaoka-Taguchi et al., 1999; Parisi and Lin, 1999).

There were two EST sequences in *Platynereis dumerilii* 48hpf cDNA library that yielded a match by the Blast search with other pumilio homologs. The clones that had a low similarity to pumilio proteins were IB0AAA21DE01, and IB0AAA34DE11 (sequence analysis by Raible et. al., unpublished). I investigated the clone IB0AAA21DE01, which showed high affinity with other pumilio homologs. The 5' end of the ORF was sequenced and showed sequence similarity to the 5' end of the mouse, fly and human homologs. The PUF domain of pumilio homologs resides at the C-terminus. This domain has not yet been sequenced for *Platynereis pumilio*. Although the 3' end of the clone had been sequenced, the sequence was too short to overcome the 3'UTR. I constructed a phylogenetic tree with the AA sequence of the 5' end of the IB0AAA21DE01. The phylogenetic tree in Figure 36B shows that *Pdu*-pumilio homolog does exist in the polychaete *Platynereis dumerilii* *Platynereis* pumilio (red) clusters together with other members of the PUF family. For this tree, I used the plants homologs of this protein family as outgroup. The amino acid sequence alignment can be seen in the appendix, chapter 6.1.4. Thus, another member of the posterior genes known to have a role in germline development in *Drosophila* exists in this polychaete. For further conclusions it will be necessary to perform WMISH more stages, to localize the proteins and get insight into the function of this genes by gain- and loss of functional studies.

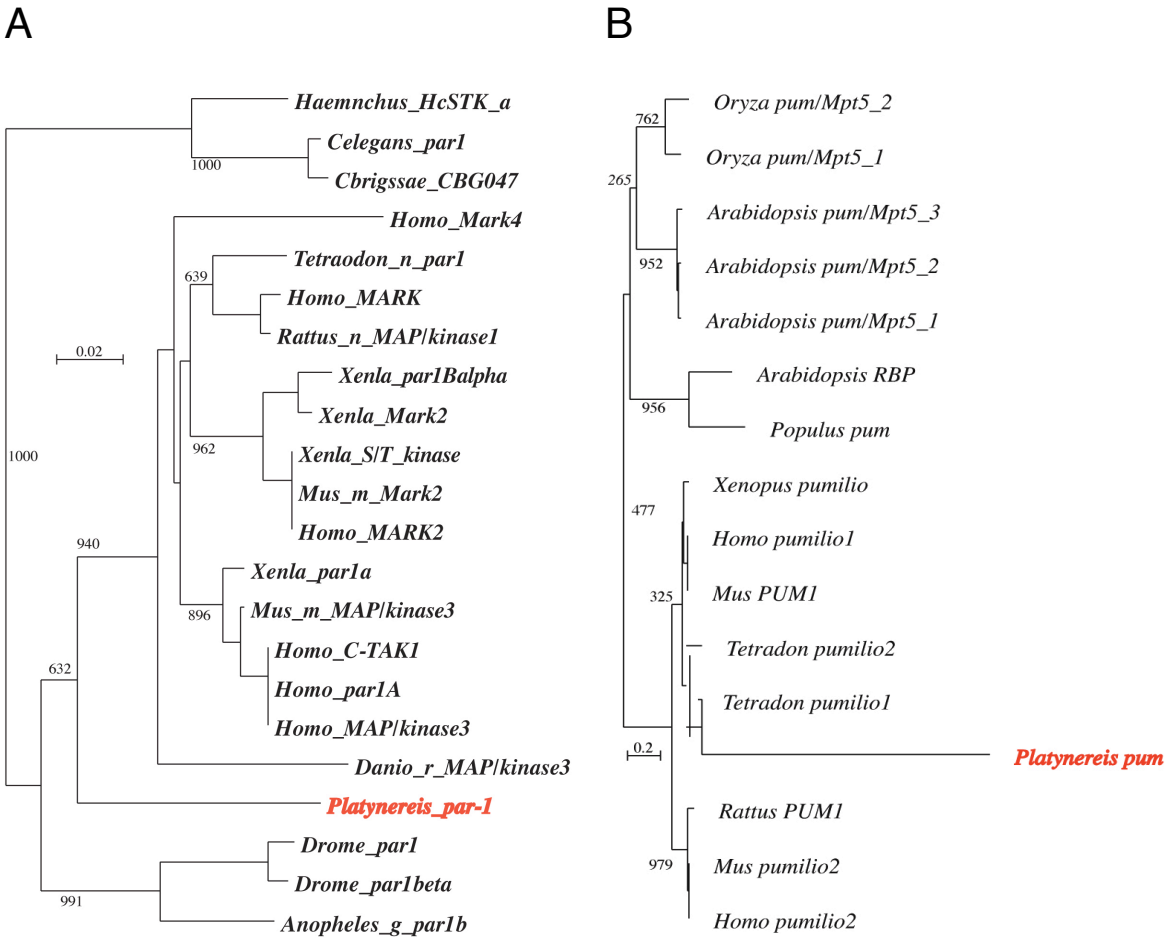


Figure 36. Phylogenetic analysis of par-1 and pumilio homologs

(A) *Platynereis* par-1 (red) is placed at the junction before the vertebrate homologs appeared. The nematode par-1 proteins were taken as outgroup. (B) The phylogenetic tree shows *Platynereis* pumilio (red) as a true homolog of its family, it clusters together with the vertebrate puf proteins. Members of the puf family in plants were taken as outgroup. The trees were constructed using the Neighbour Joining method. Bootstrap values (numbers 1 to 1000) are indicated at branch points.

3 DISCUSSION

3.1 Importance of studying the development of the germline in the polychaete

Germ cells play an essential role in the preservation of sexually reproducing multicellular organisms. Despite the diversity of Metazoan body plans and reproductive strategies, there are basically two principles of establishing the precursors of the germ cells (see introduction for more details). The maternal inheritance mode occurs in *D. melanogaster*, *C. elegans*, *X. laevis*, zebrafish and chick. The embryonic inductive mode occurs in the mouse, *Hydra*, the axotl and many other organism (reviewed by Extavour and Akam, 2003). This second mode of PGC specification was proposed to be the most ancestral one since most organisms studied to date use it.

The significance of studying the germline development in *Platynereis* derives from the lack of precise knowledge about the origin of the germ lineage in polychaetes. The present study describes the time and the location of primordial germ cell formation, it follows the germline development and reports the existence and/or expression pattern of several genes, which are involved in germline development in other organisms in *Platynereis* larval stages.

I mention in my introduction the efforts being made by several groups of developmental biologists to establish *Platynereis dumerilii* as a model system for evolutionary studies. The incorporation of *Platynereis* as a model system presupposes investigating the main developmental processes of this organism, including the origin and development of its germline. Having accurate information of the position and timing of the formation of the PGCs will allow us to apply molecular techniques for functional analysis of genes of interests. In addition to this, in the near future, information on the genome will enhance the possibility of finding promoters. Making use of promoters will enable constructing transgenic lines, which will allow us to trace the germline with a construct containing a fluorescent protein, as it was done for medaka (Tanaka et al., 2001). These transgenic lines could then be useful to monitor germ cell development of this polychaete, in the background of gene loss- and gain of function experiments.

In the following, I will summarize and discuss the most interesting conclusions of my work. I will first review the results obtained about *Platynereis* germline development in

comparison to the information reported from other species. Taken this in consideration, I will then discuss the possible evolutionary implications of germ cell determination in *Platynereis*.

3.1.1 Vasa, a suitable marker for tracing the germline in Platynereis

As exposed in the introduction, the first part of my thesis is aimed at tracing the germ cell precursors, from their origin to differentiation into gametes. *Vasa* is essential for germline development and has been used as a germ-line specific marker in several species from simple organisms, such as cnidarians (Mochizuki et al., 2001), to highly evolved ones, such as mice (Fujiwara et al., 1994) or humans (Castrillon et al., 2000). As described in the results, I cloned and studied the expression pattern of *Platynereis vasa*.

In *Platynereis* oocytes *vasa* mRNA is found in the cytoplasm, and in early cleavage stages it is ubiquitously expressed (Figure 7). It is possible that the mRNA is later degraded, except in few cells in the posterior growth zone of the trochophora larva (Figure 10E&F), from which four cells will become primordial germ cells (Figure 39F&G) and migrate to the anterior segments remaining there until they proliferate, and eventually differentiate into gametes. The pattern of *Platynereis vasa* mRNA expression is similar to the one observed in medaka, where vasa was used as a marker for tracing the germline. In medaka oocytes *vasa* mRNA is found in the cytoplasm, and in early developmental stages it is ubiquitously expressed and disappears during gastrula stages; later, *vasa* mRNA is again detected in the late gastrula stage and specifically in PGCs (Shinomiya et al., 2000).

3.1.1.1 *Platynereis vasa* codes for conserved domains characteristic of its protein family

In the following paragraphs, I will analyze the main conserved domains of vasa protein in *Platynereis* starting from the N-terminus to the C-terminus. *Platynereis vasa* codes for RGG motifs at the N-terminus. These motifs are found in zebrafish and *Drosophila vasa* homologs. They are required for subcellular localization of vasa protein in perinuclear granules (Liang et al., 1994; Wolke et al., 2002). The existence of these RGG motifs in *Platynereis vasa* and the detection of vasa protein in a perinuclear ring in the oocytes suggest a similar role of these motifs for the polychaete. The next interesting domains in *Platynereis vasa* are the CCHC-type zinc fingers at the N-terminus. This type of zinc fingers is known to bind RNA. The same CCHC-type of zinc fingers is found in vasa

homologs of *Hydra* (Cnvas1), *C. elegans* (GLH-1), *Ciona intestinalis* (Ci-DEAD-1) and in the oyster *Crassostrea gigas*. Interestingly, this has not been reported for any vasa protein in insects nor in vertebrates. The function of these zinc fingers in vasa proteins still needs to be elucidated, although taking in consideration that they are known to bind RNA, I suggest that vasa homologs that encode these CCHC domains have a higher specificity to some RNA targets. As far as the evolutionary conservation of these zinc fingers is concerned, their presence in vasa homologs in species belonging to the three groups of the Bilateria and in the cnidarians, makes it unlikely to belief that they evolved four times independently in each of these groups. Thus, this is an ancestral feature in the vasa homologs.

RNA helicases are proteins that unwind secondary structures in RNA molecules. Most putative RNA helicases have a role in one of two cellular processes, protein synthesis or RNA splicing. These include the assembly of ribosomes and initiation complexes of the protein synthesis machinery and the assembly/disassembly of spliceosomes and the actual splicing steps in the pre-RNA splicing machinery (Schmid and Linder, 1992; Wassarman and Steitz, 1991). ATP-dependent RNA helicase activity has been demonstrated in vitro for the cellular proteins elF-4A, an eukaryotic translation initiation factor (Rozen et al., 1990) and P68, a nuclear protein (Hirling et al., 1989). These and other proteins have been assigned to the ATP-dependent DEAD-box protein family of RNA helicases on the basis of eight conserved domains (Schmid and Linder, 1992). Four of these domains are known to be involved in known functions of the DEAD-box proteins (Pause and Sonenberg, 1992). *Platynereis vasa* codes for these eight conserved domains, which are characteristic for vasa-like proteins (Komiya et al., 1994; Liang et al., 1994). The five domains that are involved in ATP binding and cleavage: AQTGSGKT, PTREL(V/A), GG, TPGR, LDEAD(R/E)ML, the RNA unwinding motif: SAT, and the helicase C domain: ARGID, Y(V/I)HRIGR(T/G)GR (Figure 5A and Figure 6). Therefore, *Platynereis* vasa is likely act as an ATP-dependent DEAD-box protein of RNA helicases. Furthermore, the phylogenetic analysis reveals that *Platynereis* vasa is a true homolog of the vasa protein family (Figure 5B).

The function of vasa protein has been extensively studied is *Drosophila*. Flies carrying mutations were viable but produced no embryos (Styhler et al., 1998; Tomancak et al., 1998). Females bearing hypomorphic *vasa* alleles complete oogenesis but produce

embryos lacking germ cells and posterior segments. This indicates an essential role for vasa in germ cell formation as well as in posterior segment formation (Schupbach and Wieschaus, 1986). The protocol to achieve such mutations in *Platynereis* is not yet established. The following chapter will interpret *Platynereis vasa* mRNA expression and deduce some of its possible function by comparison with other genes at corresponding stages.

3.1.1.2 Primordial germ cells in *Platynereis* have a mesodermal origin

There is hardly any information about the origin of germ cells for the group of the lophotrochozoans. It has been proposed for molluscs, oligochaetes and polychaetes that germ cells originate from the mesoderm. This conclusion is based on the recognition of PGCs by their histological characteristics, which are oval or have a spindle like shape, granular cytoplasm containing perinuclear ribonucleotide particles and a large round nucleus with peripheral chromatin aggregations (Bürger, 1891; Hanske, 1989; Lieber, 1931; Malaquin, 1924; Meyer, 1929; Nusbaum, 1908; Pfannenstiel and Grunig, 1982; Woods, 1931). The earliest study on the germline in polychaetes traced its origin using morphological traits (Malaquin, 1924). Malaquin (1924) observed in *Salmacina distery* that at 16-cell stage of development, the 4d blastomere divided into two MG cells, which divided again giving rise to the mesodermal precursor “M” and the precursors of the germ cells “G”. I investigated that the germ cells in *Platynereis dumerilii* also derive from the MG blastomere - described as the primary mesoblasts by Wilson (1892). I want to emphasize that each of the primary mesoblasts bud forth a small cell, the secondary mesoblast (the “m” cell), which according to Wilson (1892) contains bluish-black pigment-granules. The two secondary mesoblasts gave rise to the area designated as pigmented area. The pigmented area is the posterior growth zone of the trochophora (Figure 8E,H&K and Figure 10A&F). Thus, the localization of the pigmented area described by Wilson (1892) followed partially *Platynereis vasa* expression pattern. A mesodermal origin of germ cells has also been suggested for the leech *Helobdella robusta*, in which *Hro-nanos* positive cells arise in a segmentally iterated manner form the M lineage (Kang et al., 2002), and these cells correspond to the cells that were previously proposed to be the PGCs (Bürger, 1891; Weisblat and Shankland, 1985).

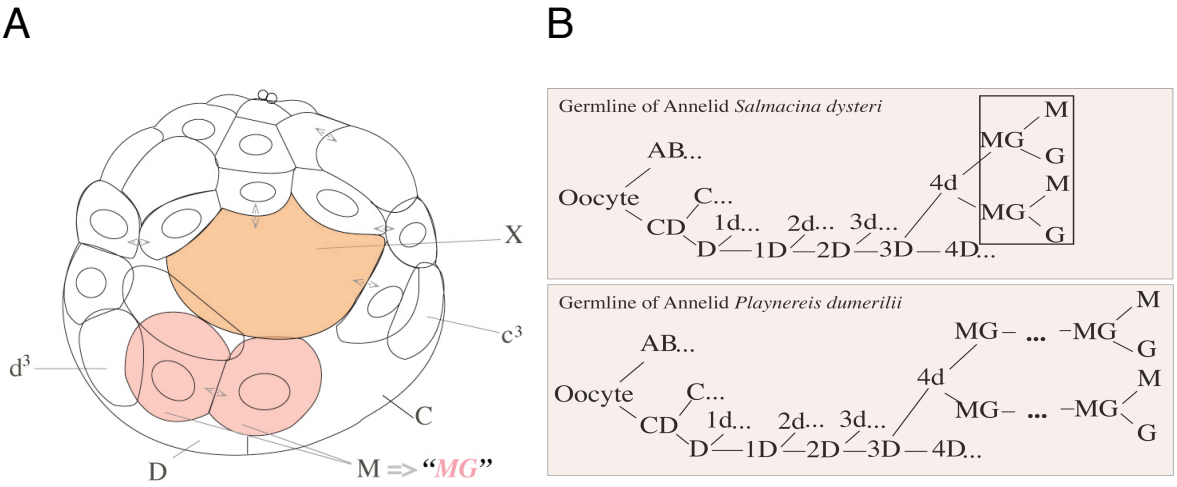


Figure 37. Mesodermal lineage of germ cells in polychaetes.

(A) 58-cell stage of *Nereis* embryo (drawing modified after Wilson, 1892): The second division of the second somatoblasts "M" (which is the 4d blastomere) into two equal sized cells primary mesoblasts *M, M* (pink, and represent the MG cells in B). The X blastomere is the first somatoblasts (which derives from 2d), the precursor of the ventral plate. MG blastomere will give rise to the mesodermal and presumably germline lineage. (B) The germ line of *Salmacina distery* derives form the 4d, which gives rise to the MG blastomeres. MG cells divide into the mesodermal precursor "M" and germ cell precursor "G" cell (Malaquin, 1924). For *Platynereis dumerilii* the difference is that the MG blastomeres do not divide into M and G cell directly, but only after several divisions. Four PGCs are specified later in the trochophora.

In summary, *Platynereis dumerilii* is an example of an organism, as it is *Helobdella robusta*, that in contrast with *Drosophila*, *Xenopus* and *C. elegans*, does not segregate its germline from somatic cells early in development, but rather its germline has a mesodermal origin and is specified later in embryogenesis. I propose that inductive processes specify the primordial germ cells in *Platynereis dumerilii*.

3.1.1.3 Restriction of vasa to the germ cell lineage

By which mechanism is *Platynereis vasa* restricted to the germ cell lineage? The analysis presented in this thesis does not allow answering this question. Yet the fate of the *vasa* homolog in zebrafish suggests the mechanisms to restrict *vasa* into the germline. In zebrafish, *vasa* mRNA is localized to the germ plasm (Knaut et al., 2000; Yoon et al., 1997), while the maternal vasa protein is initially distributed uniformly in the embryo and its allocation becomes restricted to the PGCs only later in development (Braat et al., 2000; Knaut et al., 2000). Both, its mRNA and protein are segregated into the PGCs by a posttranscriptional degradation-protection mechanism (Wolke et al., 2002). A similar mechanism might act in *Platynereis vasa* processing, since in this polychaete the transcript were restricted to the PGCs later in larval development. Furthermore, since the existence of

a 'nuage' like structure been reported in the oocytes of this polychaete (Fischer, 1975), it could have been anticipated that the specification of germ cells would dependent on the inheritance of maternal determinants stored in the germ plasm, rather than due to inductive processes, which would take place later during embryogenesis. In *Platynereis* both maternal *vasa* mRNA and vasa protein were detected in oocytes. Later in embryogenesis, they were both allocated to the growth zone, the region from which the four PGCs arise. The mRNA was degraded from the growth zone, and subsequently the protein was restricted to the four PGCs. The mRNA and protein were segregated to the PGCs, probably by a posttranscriptional degradation-protection mechanism mediated by its RGG motifs (discussed above). Since the PGCs of *Platynereis* develop only after the MG has gone through several divisions (Figure 37A and B), and are segregated in the three days old worm (N. Rebscher, personal communication and see Figure 39F), I conclude that the PGCs in this polychaete are specified by extrinsic signalling, thus much later in development.

3.1.1.4 *Vasa* expression in juveniles and mature worms

Platynereis dumerilii does not develop gonads, and gametogenesis takes place in the coelomic cavity (Hauenschild and Fischer, 1969). Oogonia and spermatogonia appear in the coelomic cavity as syncytial clusters of up to 64 cells, which are surrounded by a sheath of somatic cells (Fischer, 1974; Fischer, 1975; Hanske, 1989; Hauenschild and Fischer, 1969). The oogonial and spermatogonial clusters were reported to develop from the dorso-lateral coelothel (Hanske, 1989). These clusters fall apart into single spermatogonia or oogonial, floating in the coelomic cavity until the worm is ready to spawn (see purple box in Figure 39). The results reported here on *Platynereis vasa* expression in adult worms (Figure 11 - Figure 13) together with the immunohistochemistry investigations done by N. Rebscher (personal communication), revealed gonial clusters and oocytes in the dorso-lateral parapodial region in juveniles older than 1-1 1/2 month (Figure 11C-G and Figure 16). *Vasa* positive oocytes were later filling the coelomic cavity (Figure 11G, H, K & L), and no *vasa* mRNA was detected in spermatozoids. Thus, these data corroborate earlier observations by Fischer (1975) and Hanske (1989).

There is no *Pdu-vas* mRNA expression in PGCs migrating from the growth zone. *Pdu-vas* mRNA was degraded in the growth zone after 6dpf, while its protein was present there for longer time. The immunohistochemistry experiments showed that four cells containing

vasa protein migrated from the growth zone to the base of the parapodia of the 2nd larval segment. These four cells were in adult stages located in the region between the 4th & 5th adult segment (Nicole Rebscher, personal communication). Once these cells started to proliferate, *vasa* mRNA was again detected by WMISH (Figure 11A and Figure 38A and B). Some of these cells became gonadal clusters at this position, and others might have migrated to the posterior and then have developed into gonadal clusters. Predators often bite the tail of this polychaete and storing its PGCs in the anterior segments while growing, and not somewhere else, might have developed as a mechanism for guarding these cells -that will eventually give rise to the next generation- from predators, thus ensuring the preservation of the species. One question remains to be answered, and this is whether the descendants of the four PGCs proliferate, do they migrate to posterior segments and then differentiate into gametes, or whether some somatic cells in each segment trans-differentiate into PGCs, proliferate in that location and then differentiate.

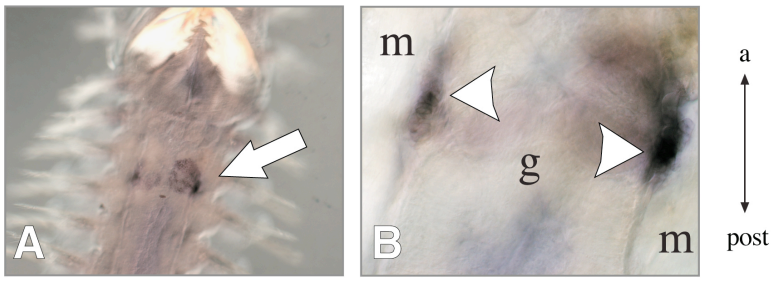


Figure 38. Proliferation of PGCs

(A) In juveniles, PGCs proliferate forming a cravat like structure between the 4th and 5th segment- white arrow. These cells form a layer between the muscles [m] and the gut [g], as shown in (B- white arrowheads).

A summary of both *Pdu-vas* mRNA (blue) and vasa protein (green) distribution throughout the germline development in *Platynereis* is depicted in Figure 39. In the unfertilized oocyte Vasa protein forms a ring around the nucleus while *vasa* mRNA is ubiquitously expressed (Figure 39A). A different distribution of vasa protein is seen at 48hpf, and it is detected at the growth zone (Figure 39E). Meanwhile, the transcript was degraded, but remaining in the stomodaeum and growth zone through larval stages -24-72hpf- (Figure 39B-F). At 4dpf four PGCs expressing vasa protein migrate from the growth zone. They remain at the base of the parapodia of the 2nd and 3rd larval segments (Figure 39F&G). At later stages these cells are detected in the region between the 4th and 5th segment (Figure 39H), where they proliferate (Figure 38A&B). Those PGCs most probably migrate to the posterior regions and differentiate giving rise to gametes (Figure 39H, I and pink box).

In conclusion, a vasa homolog exists in *Platynereis dumerilii* and its expression can be used for tracing the germline development in this polychaete.

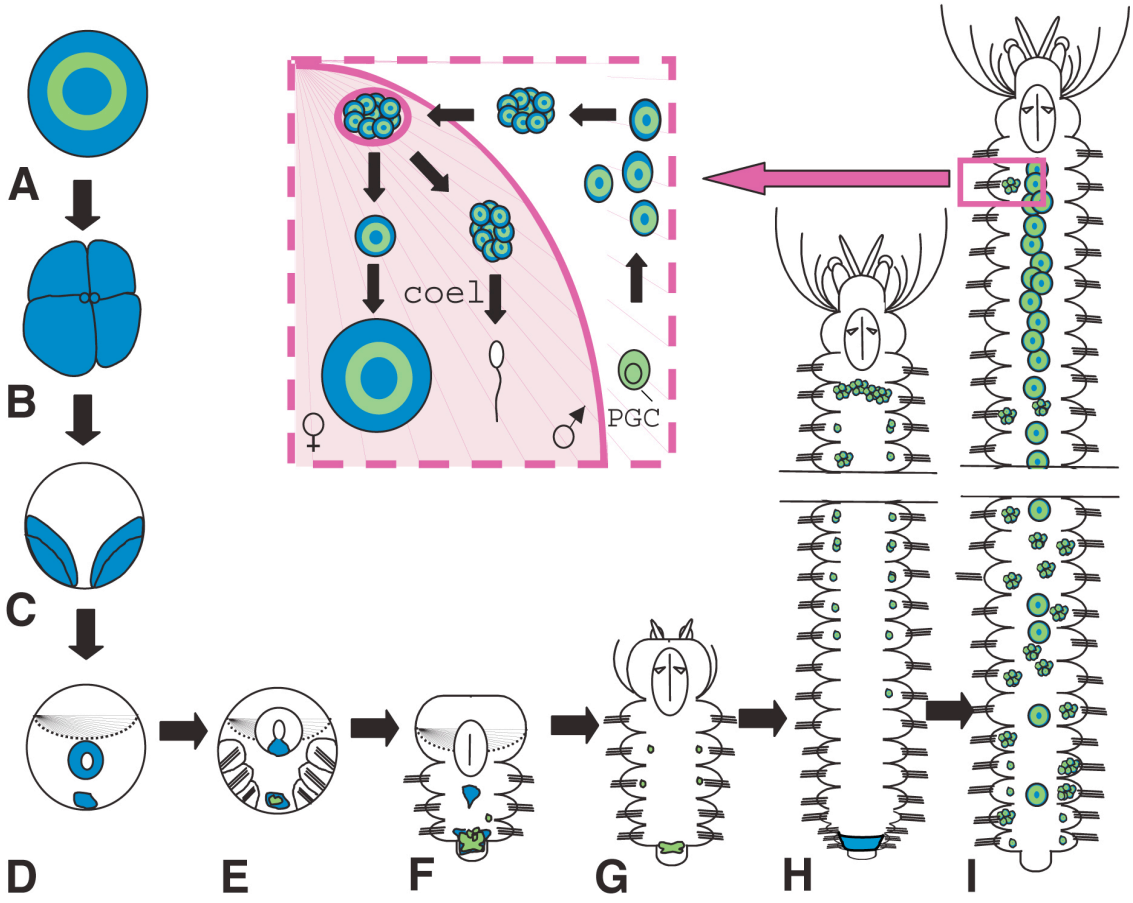


Figure 39. *Pdu-vas* mRNA and protein localization in different stages of development

(A) In oocytes, maternal *Pdu-vas* mRNA is ubiquitously distributed. Vasa protein forms a perinuclear ring. (B) At the 4-cell stage, *Pdu-vas* mRNA distribution is ubiquitous. The protein location at this stage is unresolved, although it is detected by immunoblot -the same situation for (C) and (D). (C) At 15hpf, after gastrulation, *Pdu-vas* mRNA is expressed in vegetal hyposphere. (D) The 24hpf old trochophora larvae shows *Pdu-vas* mRNA in the developing stomodaeum as well as a in the posterior growth zone [GZ]. (E) At 48hpf Vasa protein is localized to the GZ, being there co-expressed with *Pdu-vas* transcript. High levels of transcript are detected in the stomodaeum. (F) At 72hpf to 4dpf, the posterior growth zone exhibits a butterfly like pattern for both Vasa protein and mRNA. There is *Pdu-vas* mRNA staining in the deep midline of the worm. Vasa protein is distinguishable in four cells, which at this stage are detaching from the growth zone. (G) At 1mpf, Vasa protein is still detected in the posterior GZ and in 4 primordial germ cells [PGCs] that are located in the 3rd and 4th segments, at the base of the parapodia. No *Pdu-vas* mRNA is detectable at this stage. The four PGCs are later found in the border between the 3rd and 4th segment. (H) Apparently, in juvenile worms longer than 20 segments, the density per volume of cells containing both Vasa protein and *Pdu-vas* mRNA displays a gradient (but not the concentration of Vasa molecules), with more cells at the anterior. A neck like structure expressing both Vasa protein and *Pdu-vas* mRNA is detected between the 4th and 5th segment. Gonial clusters that detach from this region float freely in the coelomic cavity. Oocytes continue expressing both Vasa protein and mRNA, while there is no *vasa* transcript or protein in spermatocytes. In two months old juveniles of 15 to 36 segments, *Pdu-vas* mRNA expression is found in the growth zone, excluding the pygidium. (Drawing modified after Nicole Rebscher).

3.1.2 Nanos is expressed in primordial germ cells

As I mentioned before (see introduction), a first aim of my thesis was to investigate the germline development of *Platynereis*. *Nanos* was the second candidate for this investigation, since it has been used as a germ cell tracer in the leech and in the hydrozoan *Podocoryne carnea*. In leech, the authors followed the maternal and zygotic expression of *nanos* to examine the origin of the PGCs, they found that the PGCs arise from segmental mesoderm (Kang et al., 2002). In the hydrozoan, *Podocoryne carnea* two *nanos* homologs exist. *Nanos* expression is widely expressed in the developing medusa and progressively restricted to a germline specific pattern at the terminal stage of medusa development, corresponding to the known late distinction between germline and soma in hydrozoans (Torras et al., 2004). In addition to these two examples, *nanos* is involved in germ cells maintenance or/and their proper migration to the gonads in several species (discussed below).

The phylogenetic analysis shows that *Platynereis* nanos clusters together with mouse nanos2 and nanos3, and human nanos2 and nanos3 (Figure 22C). It is not surprising that *Platynereis* nanos is more likely to cluster with the mammalian homologs rather than to the leech one, since leech is a derived species for its phylum –the annelids. Moreover, this is not the first example of a lophotrochozoan gene that clusters with a stronger affinity to its human or/and mouse homolog (Raible and Arendt, 2004).

In *Drosophila* nanos two zinc-binding domains are required for high affinity binding to RNA and mutations in either of these two sites *in vitro* abolish nanos translational repression activity *in vivo* (Curtis et al., 1997). The similarity among nanos-related proteins is restricted to these putative CCHC double zinc finger motifs. The amino acid sequences in the regions both N-terminally and C-terminally to the zinc finger domain are not conserved among different phyla. *Platynereis nanos* encodes these double zinc finger motifs (Figure 22B), and based on this similarity functional conservation is likely. Remarkably this same type of CCHC zinc fingers is found in *Platynereis* Vasa (discussed in the previous chapter). The CCHC type of zinc fingers is known to bind RNA, in the case of nanos protein, preventing for example *hunchback* translation. For this reason, nanos protein is known as a translational repressor.

The *nanos* homolog in *Drosophila* and *C. elegans* are a maternally contributions to the embryo and a component of the germ plasm and polar granules, respectively. It is not

required for PGCs formation nor for early specification of the germ cell fate, but its function is the maintenance of the germ cells state and to promote incorporation of the germ cells into the gonad (Deshpande et al., 1999; Forbes and Lehmann, 1998; Kobayashi et al., 1996; Subramaniam and Seydoux, 1999). Recently, it has been demonstrated in *Drosophila* that *nanos* is directly and continuously required for both the establishment and the self-renewal of germinal stem cells (GSCs). *Nanos* does so by preventing primordial germ cells (PGCs) and GSCs from prematurely entering the oogenic pathway. Translational regulation maintains stem cell properties by preventing the precocious activation of a differentiation pathway. In *C. elegans* three genes exist (*nos-1*, *nos-2* and *nos-3*) which encode a putative zinc finger-binding domain, similar to the one found in *Drosophila nanos*. *nos-1* and *nos-2* are not generally required for PGC fate specification, but instead regulate specific aspects of PGC development. *Nos-2* mRNA is expressed in PGCs around the time of gastrulation from a maternal RNA associated with P granules, and it is required for the efficient incorporation of PGCs into the somatic gonad. *Nos-1* mRNA is expressed in PGCs after gastrulation. Nos-1 protein, synergistically with nos-2 protein, prevents PGCs from dividing in starved animals and to maintain germ cell viability during larval development. If no nos-1 and nos-2 proteins are present then germ cells cease proliferating at the end of the second larval stage and die (Subramaniam and Seydoux, 1999). The *nanos* vertebrate homologs in *Xenopus* and zebrafish are also components of the germ plasm. In zebrafish it is expressed in the PGCs and it is required for their proper migration and survival (Koprunner et al., 2001; Mosquera et al., 1993; Nakahata et al., 2001; Zhou and King, 1996).

In contrast to most organisms in which the transcript is maternally derived, *Platynereis nanos* is first detected at the mid-blastula transition around 5hpf. The mid-blastula transition marks the onset of zygotic transcription (Figure 25), thus *Platynereis nanos* is zygotically expressed. RT-PCR was performed with RNA extracts from whole embryos. At 5hpf the transcripts represent the concentration of *nanos* mRNA in the mesodermal and germline lineage (Figure 25) since the first evidence of *Platynereis nanos* positive cells in the brain is detected with WMISH at 15hpf (Figure 23C and D). Similarly, no maternal transcripts have been detected for two mice *nanos* homologs. RT-PCR and ISH experiments show that murine *nanos2* expression is restricted to the PGCs as early as embryonic stage 9.5, and is expressed in male gonads at a later stage of development,

whereas murine *nanos3* expression is restricted to the developing male PGCs at embryonic stage 13.5 (Tsuda et al., 2003).

As discussed in the previous chapter, *Platynereis* germ cells have the same origin as the mesodermal bands, since both germline and mesodermal lineage develop from the same pair of mesoblasts. Taking into account that at 12hpf *Platynereis nanos* transcript is detected in the mesoblasts (Figure 23B) and at 15hpf in the mesodermal bands (Figure 23G), I conclude that at this stages no cells have yet acquired PGCs characteristics although the function of *nanos* at this stage in the mesodermal lineage might be tightly linked with the later formation of the PGCs. The four PGCs in *Platynereis dumerilii* develop in the growth zone around 72hpf to 4dpf (Figure 24D&H and Figure 39F), and start migrating to the anterior at around 4dpf to 5dpf (Figure 39F&G). These four PGCs are identified with vasa antibody (discussed in chapter 3.1.1.4 and Rebscher and Zelada-Gonzalez, unpublished). *Platynereis nanos* positive cells are detected in the growth zone at the time when the PGCs are formed (Figure 24D). Importantly, four *nanos* positive cells are found in the growth zone at 4dpf (Figure 24H). By 6dpf no *nanos* mRNA is detected either in the growth zone with WMISH nor in the migrating PGCs, although the latter would require more investigations since detection of four individual cells with WMISH is a difficult task. My data suggests that the transcript is probably down regulated after the formation of the primordial germ cells. *Platynereis dumerilii* does not develop gonads (Hauenschild and Fischer, 1969). Nevertheless, the function of *nanos* for proper PGC migration into the gonads in other organisms could be compared with the development of the four PGCs at the growth zone and later with their migration from the growth zone to the more anterior segments, and probably to their localization at the border of the 4th and 5th adult segment before PGCs proliferation (Figure 39 and Figure 24).

In conclusion, *nanos* expression in the primordial germ cells of *Platynereis* suggests a conserved role in PGC maintenance, as described in other organisms.

3.1.3 A pumilio homolog exists in Platynereis

A second player that interacts with nanos is **pumilio**. In *Drosophila*, nanos and pumilio proteins are also involved in the migration of pole cells and in gene expression in migrating pole cells (Asaoka-Taguchi et al., 1999). A region C-terminal to the zinc finger domain has also been shown to be necessary for abdominal formation and germ cell migration (Arrizabalaga and Lehmann, 1999). Nanos and pumilio proteins may function

together in preventing the PGCs and GSCs from activating a normal differentiation pathway (Wang and Lin, 2004). There are at least eight genes in *C. elegans* genomic database related to *Drosophila pumilio* (Zhang et al., 1997). Disruption of five of these *pumilio*-related genes resulted in similar phenotypes as observed for *nos-1* and *nos-2* RNAi treated ones. In addition to the *Drosophila* data, there are two described pathways that give information about the interaction of nanos and pumilio proteins. In *C. elegans*, FBF- one of these pumilio homologs- directly binds to the N-terminal region of nanos-3 protein and the complex regulates the translation of *fem* mRNA, thereby achieving the switch from spermatogenesis to oogenesis in hermaphrodites (Kraemer et al., 1999). The human nanos-1 homolog interacts with the human pumilio-2 homolog, and both proteins are particularly abundant in human in germline stem cells in men (Jaruzelska et al., 2003). These observations reflect conservation in the interaction of both proteins in distant species. Two specific findings support an interaction also in *Platynereis*: first, *Platynereis nanos* is expressed in PGCs (Figure 24H) and second, at least one *pumilio* homolog exists in this polychaete (Figure 36B), although a co-expression of these two mRNAs still needs to be elucidated.

3.1.4 An additional feature of Par-1 is found in Platynereis

I chose to investigate *par-1* in *Platynereis* since it has a pivotal and conserved role in the germline development and in the establishment of embryonic polarity in *Drosophila* and *C. elegans*, which are two well-studied model organisms (Rose and Kemphues, 1998; Tomancak et al., 2000). Par-1 belongs to the EMK family of kinases (Plowman et al., 1999). Members of this family of kinases are known to be involved in establishing the cytoplasmic polarity in quite different organisms, from yeast to humans (Barral et al., 1999; Levin and Bishop, 1990; Parsa, 1988). Studying the expression pattern and function of this gene in *Platynereis* could help us to understand the role of an additional player that is involved in germline development and axis formation, and its conservation in several phyla.

In *Drosophila* and *C. elegans*, par-1 is necessary to stabilize the germ plasm proteins in the posterior of the embryo. In *Drosophila* par-1 acts by direct phosphorylation of oskar protein (Riechmann et al., 2002) and regulating the dynamics of the microtubules (Shulman et al., 2000), while the par-1 homolog in *C. elegans* stabilizes pie-1 through two intermediates (mex-5 and mex-6 proteins), which have to be down regulated in the

posterior end of the embryo to achieve the stabilization of pie-1 (Cuenca et al., 2003). *Drosophila par-1* mutants failed to localize *oskar* mRNA to the posterior pole of the oocyte, resulting in embryonic patterning effects. The posterior localization of *oskar* mRNA is the key step for the assembly of polar granules, because oskar protein recruits components of the polar granules such as vasa protein (Ephrussi et al., 1991; Ephrussi and Lehmann, 1992; Kim-Ha et al., 1991).

As shown in this work, *Platynereis* has a true *par-1* homolog, which codes for the typical par-1 protein kinase domain and clusters together with par-1 homologs of other species (Figure 33 and Figure 36A). The mammalian Par-1 homologs belong to the Marks proteins - seen in the phylogenetic tree. They are known to regulate the dynamics of microtubules (Drewes et al., 1997; Ebneth et al., 1999).

Moreover, *Platynereis par-1* is a maternally transcribed gene (such as the homologs in *Drosophila* and *C. elegans*), since it was up-regulated in fully matured females and was present at high levels in unfertilized oocytes; the transcript was rapidly degraded to lower levels after 5hpf (Figure 35).

To elucidate the role of par-1 in cytoplasmic partitioning and asymmetric cell division in *Platynereis* early embryogenesis, it would be useful to localize *par-1* mRNA and protein throughout the first cleavage stages and this information is not yet available.

The function of par-1 in other organisms is not restricted to early embryonic development. In *C. elegans*, par-1 was required for morphogenesis of the *C. elegans* vulva (Hurd and Kemphues, 2003). *Platynereis par-1* is expressed in the most anterior region of the brain (Figure 34C and D). This suggests its role in brain development in this polychaete.

In conclusion, I report in this thesis the existence of a true par-1 homolog in *Platynereis*. It is present at the right time and place to function in A/P patterning in the polychaete (Figure 35). In addition to this, par-1 appears to be involved in brain development of the young worm. Further experiments in early cleavage stages might elucidate par-1 involvement in cytoplasmic partitioning, thus providing more information about the possible existence of molecular assemblies like the polar granules of *C. elegans* and the germinal granules in *Drosophila*.

3.2 Relationship between the germ cells and undifferentiated cells (totipotent cells)

Totipotency, a term most likely used for the first time in 1909, refers to the capacity of cells to generate or regenerate an entire new organism (Mish, 1988). Are primordial germ cells in general totipotent, meaning that those cells can differentiate to any kind of cell, or are they already determined and restricted to give rise to sperms and oocytes?

In many organisms, morphological and molecular connection has been found between the germ cells and pluri or totipotent cell. Recent evidence shows that the closest in vivo equivalent of an embryonic stem (ES) cell is the early germ cell (reviewed by Zwaka and Thomson, 2005).

The i-cell lineage in *Hydra* consists of multipotent stem cells and their differentiation products. These i-cells give rise –among others- to germ cells (Bode, 1996; Mochizuki et al., 2001). In another cnidarian, *Hydractinia echinata*, recent studies demonstrated that the i-cell lineage comprises totipotent stem cells (Muller et al., 2004). No germ plasm or chromatioid body like structures have been reported for i-cells in cnidarians, so far.

Cultured ES cells in mice were considered pluripotent rather than totipotent, because of the failure to detect germline cells under differentiating conditions, but lately it was shown that those cells in culture can develop into oogonia and oocytes that enter meiosis, recruit then adjacent cells to form follicle-like structures and develop into blastocysts (Hubner et al., 2003).

Planarians are known for their regenerative ability. This ability has been considered to reside in the totipotent somatic stem cells called neoblasts. Neoblasts, described by their morphological characteristics, are small in size (about 10 μm), round to ovoid in shape, and possess a high nucleus/cytoplasm ratio. Their cytoplasm contains a few mitochondria, many free ribosomes, and few other organelles (Hori, 1982; Morita, 1967; Pedersen, 1959) .These characteristics suggest that the neoblasts are undifferentiated cells. The most noticeable feature of the neoblasts is the “chromatoid body” in the cytoplasm detected by electron microscopic observations (Coward, 1974). The chromatoid body is also found in cytoplasm of the germ cells and it is the reminiscent of the germinal granules.

Furthermore, this structure was also found in regenerative cells which were defined as ‘differentiation” cells derived from the neoblasts. The chromatoid bodies decreased in

number and size during cell differentiation and were no longer detected in terminally differentiated cells (Hori, 1982; Hori, 1992).

This work reports data in *Platynereis* on *pl10* and *piwi*, which are two genes that are known to be expressed in multi- and totipotent cells (Mochizuki et al., 2001; Shibata et al., 1999), and stem cells (Benfey, 1999; Cox et al., 1998). In *Platynereis*, *pl10* expressed in the germline (discussed in 3.1.2) and *piwi* overlaps with the expression pattern of *vasa* in the growth zone (discussed in 3.2.3 and see Figure 42A&D and Figure 43)- and the region where the PGCs are formed- at different stages of development. In addition, both *vasa* and *pl10* are expressed in regenerating tails (see chapter 2.1.3). Thus, molecular markers for totipotent cells are expressed in the germline lineage in this polychaete. These data supports the view that primordial germ cells could be totipotent similar to i-cells, neoblasts and ES cells in other organisms, although this characteristic of germ cells is easier to identify in those organisms in which regeneration is a common and essential feature for their subsistence, such as *Platynereis*.

3.2.1 Vasa expression in Platynereis suggests its ancestral role in not terminally differentiated tissue

In early developmental stages *Pdu-vas* mRNA is expressed in the growth zone, in cells that have the potential to become both mesoderm and germ cell. *Pdu-FgfR* is a marker for undifferentiated mesoderm, and is co-expressed with *Pdu-vas* in the growth zone. *Pdu-FgfR* is also expressed in the mesodermal tissue between the chaetal sacs, where no *Pdu-vas* positive cells are detected (Figure 40A and B). There is no *Pdu-vas* expression in mesodermal cells expressing *Pdu-twist*, or in muscles expressing *Pdu-MyoD* and *Pdu-Mfe2* mRNA staining (Figure 40C to E). Thus, I conclude that once these cells (the “MG” cells) are committed to the mesodermal lineage (“M”), *Pdu-vas* expression is turned off. Similar results were presented for the hydrozoans using *nanos* homolog as a germline marker, in which, one of the two homologs (*Cnnos1*) is specifically expressed in multipotent stem cells and germline cells, but not in somatic cells, and its expression ceased once the multipotent stem cells enter the somatic pathway (Mochizuki et al., 2000).

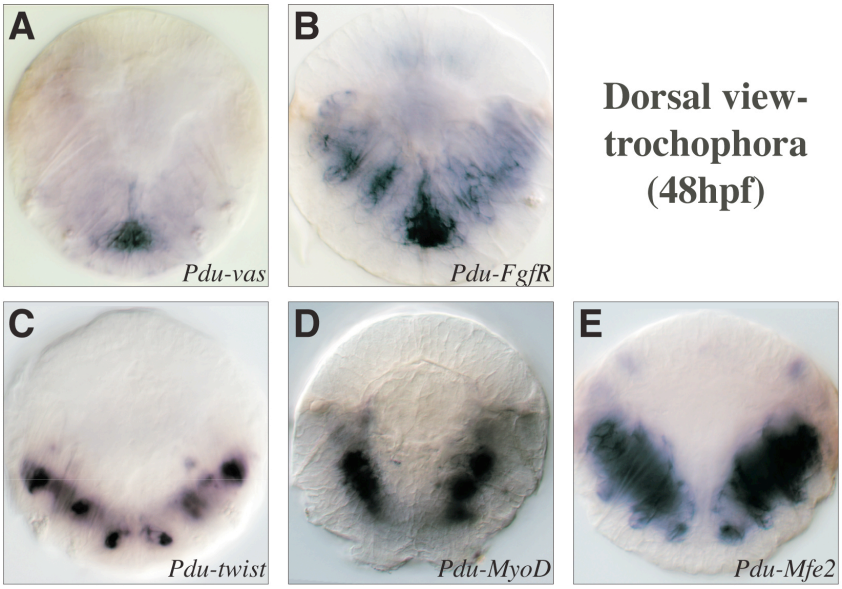


Figure 40. Comparison of *Pdu-vas* mRNA expression with different mesodermal markers

Dorsal view of trochophora larval stage [48hpf]: (A) *Pdu-vas* mRNA expression in the GZ, a marker for germ cells and cells committed to a mesodermal lineage. (B) *Pdu-FgfR* mRNA expression in GZ and in the mesoderm of the tissue between the chaetal sacs, a marker for undifferentiated mesoderm. (C) *Pdu-twist* mRNA expression in mesodermal cells. (D) *Pdu-MyoD* transcript is expressed in muscle cells. (E) *Pdu-Mfe2* mRNA expression in muscles cells. (Pictures B to E are courtesy of Patrick Steinmetz)

In summary, the function of vasa in this polychaete is not just restricted to germline formation, but also plays a role in growth zone, regenerating tails and in the formation of the stomodaeum (see below). A dual role has been also suggested for *Hydra*, in which *vasa* is expressed in large i-cells (Mochizuki et al., 2001). The i-cell lineage in *Hydra* consists in multipotent stem cells and their differentiation products. A subpopulation of the large i-cells is considered to be the stem cells. Neoblasts are totipotent stem cells in planarians and express *vasa*-related genes (Shibata et al., 1999). The growth zone in *Platynereis* is the region where new segments originate and expresses *Pdu-vas* in the trochophora and young worms (Figure 8D-L and Figure 10E-G), adult worms (Figure 11I) and in regenerating tails (Figure 20A&F). Therefore, I propose that *vasa* positive cells in the growth zone have multi- or totipotent characteristics, as have the i-cells in *Hydra* and neoblasts in planarians.

Conclusions:

- *Vasa* expression patterns to suggest that its function is not restricted to germline development, but also plays a role in tissue before terminal differentiation and regeneration and in the formation of the stomodaeum (see below).

- *Platynereis vasa* is expressed in putative multipotent stem cells of the growth zone including the primordial germ cells of the trochophora larvae, but not in terminally differentiated somatic cells, and it is down-regulated once the multipotent stem cells enter the somatic pathway or give rise to the four PGCs. *Vasa* is again up-regulated during gametogenesis.

3.2.2 Comparison of Pdu-vas and Pdu-pl10 expression during the onset of gametogenesis

Pl10-related proteins belong to the DEAD-box protein family of RNA helicases. It has been proposed that *vasa*-related genes arose by duplication of a *pl10*-related gene before the appearance of sponges but after the separation of fungi and plants (Mochizuki et al., 2001). The PL10- related proteins are highly conserved among a diverse range of eukaryotes from yeast (Chuang et al., 1997; Kawamukai, 1999) to plants (Lin et al., 1999) and animals (Gee and Conboy, 1994; Gururajan et al., 1991; Leroy et al., 1989; Olsen et al., 1997) in their amino acid sequences. In addition to this, *DED1*, one of the PL10-related protein in yeast, may be required for translational initiation of almost all mRNAs, and the mouse PL10 can rescue the phenotype of a yeast mutant with chromosomal *ded1* deletions (Chuang et al., 1997). This emphasizes the functional conservatism of these proteins. The expression pattern of *Pdu-pl10* is similar to *Pdu-vas*, although *Pdu-pl10* expression is broader in *Platynereis* larval stages (Figure 8 and Figure 16). This might mean that more cell types need Pl10 for proper function, possibly for translational initiation of quite a few mRNAs. Its expression in regenerating tails and growth zone suggest its up-regulation in highly proliferating tissue or/and not yet terminally differentiated cells that will eventually give rise to the new segments. *Pl10*-related genes are expressed in totipotent cells in *Hydra* and planarians (Mochizuki et al., 2001; Shibata et al., 1999). This strengthens my suggestion of *Pdu-pl10* positive cells being “not yet” terminally differentiated. *Platynereis pl10* expression is in agreement with data reported for its homolog in *Xenopus* (*An3*) in the sense that its zygotic expression is ubiquitously distributed throughout the embryo (Gururajan et al., 1991).

In the region between the 4th and 5th segment, a collar like structure formed by proliferating PGCs, which later became gonadal clusters (Figure 41A-zone I). These cells expressed *Pdu-vas* and *Pdu-pl10*. In the following segments, high levels of both transcripts were detected in the peritoneum and in the coelomic cavity of the parapodia (Figure 41A-zone

II). In zone III and IV, cells expressing *Pdu-vas,* and *Pdu-pl10* respectively, were detected in the parapodia. In the case of *Pdu-vasa*, zone III showed single *vasa* positive cells along the gut, these cells had an elongated shape and contained a big nucleus, and these cells might be primordial germ cells (Figure 41A, and B-left). In zone IV, round oocytes expressing *Pdu-vas* are noticeable (Figure 41A, and B- right). The difference in expression between *Pdu-vas* and *Pdu-pl10* at this stage of development is that *Pdu-pl10* is expressed in spermatogonial clusters (Figure 41B-left), in contrast to *Pdu-vas*. *Pdu-pl10* is also detected in oocytes, as well as *Pdu-vas* (Figure 41B-right). No *Pdu-vas* or *Pdu-pl10* transcript is detected in the tail (Figure 41A-zone V). Thus, in adult worms both *Pdu-pl10* and *Pdu-vasa* are expressed in a gradient manner with higher levels of expression at the anterior. This gradient reflects the amount of cells per volume and not the concentration of the transcripts within the cells.

Platynereis pl10 expression in male gonadal structures –in contrast to *vasa* expression- is not the only example reported, since the *pl10* homolog in the mouse is testes-specific (Leroy et al., 1989).

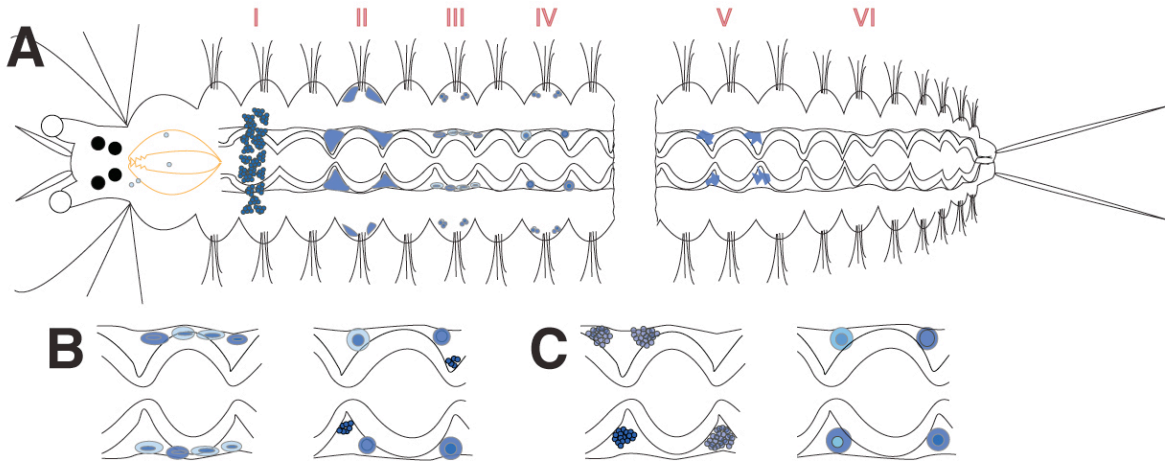


Figure 41. *Pdu-vas* and *Pdu-pl10* gradient expression in adult worms (> 45 segments)

(A) Adult worm at least older than two months, in which gonadal structures are recognizable. Zone I: region between the 4th and 5th adult segment, wherein first, the PGCs proliferate and then gonadal clusters are noticeable. Between zones II and I resides a gap of *pl10* and *vasa* mRNA expression. Zone II: pl10 and vasa transcript in cells lying between the gut and muscles, as well as in the base of the parapodia. Zone III: gonadal structures are recognizable; for *vasa*- growing and vitellogenic oocytes, seen in (B) left and right respectively, and for *pl10*- spermatogonial clusters or oocytes as shown in (C) left and right respectively.

3.2.3 Piwi expression in highly active proliferating cells

Piwi represents a class of genes required for stem cell-renewal in many organisms (Benfey, 1999). No *piwi* homolog exists in neither bacteria nor yeast, this is consistent with stem cell-renewal function of *piwi* and its specificity in multicellular organisms (Cox et al., 1998). In *Drosophila*, *piwi* encodes a nucleoplasmic protein, which is present in somatic and germline cells. It is a key regulator of stem cell division. Its somatic expression modulates the number of germline stem cells and the rate of their division, while its germline expression also contributes to promoting stem cell division in a cell-autonomous manner. This is consistent with the fact that, germline stem cells in *Drosophila* ovary have self-renewal capacity, like other stem cells do (Cox et al., 1998). By definition, all stem cells must have self-renewal capacities; otherwise they would not be stem cells, but merely progenitors.

In mammals, *piwi*-related genes are only expressed in the testes. Here, they regulate the stability of spermiogenic mRNAs, hence regulating spermatogenesis and germ cell proliferation (Deng and Lin, 2002). In the hydroid *Podocoryne carnea* it is the adult medusa that expresses *Cniwi*, the homolog for *piwi*; however, it is expressed not only in germ stem cells located in the male and female gonads, but also in somatic cells that have the ability to trans-differentiate and regenerate (Seipel et al., 2004). These cells acquire multipotent characteristics after or during trans-differentiation. This might also be the case in *Platynereis dumerilii piwi* positive cells, since in larval stages they reside in tissue that is highly proliferating and morphogenetically active, such as the stomodaeum, which during those stages is invaginating to form the mouth, and the growth zone. In addition to this, *Pdu-pl10* expression pattern in larval stages overlaps with the one of *Pdu-piwi* (Figure 42C and D). *Pdu-pl10* is expressed in undifferentiated cells (see chapter 3.1.2) and since stem cells are undifferentiated cells, this strongly supports the possibility of a conserved role of *piwi* in stem cell renewal in this polychaete.

Moreover, *piwi* is expressed in the tissue where the chaetal sacs are being formed and in the growth zone, which is the region from which new segments bud (Figure 21B-F). A comparison of *Pdu-piwi* with *Platynereis Fgf receptor* (*Pdu-FgfR*), a marker for undifferentiated mesoderm in the polychaete (P. Steinmetz, unpublished) shows that these genes are co-expressed in the distal region of the stomodaeum (Figure 42B and D), in the mesoderm between the forming chaetal sacs (Figure 21B-black arrowheads and Figure

40B) and in the growth zone (Figure 21B and Figure 40B). Thus, the expression pattern of both genes is similar and this suggests a role of piwi in the Fgf signalling pathway.

The expression pattern of *Pdu-piwi* mRNA seems to be nuclear. The latter was described for *Drosophila*, mouse and human *piwi* homologs, and it is not the case for *Drosophila aubergine*, and mouse *miwi* and *milli* - other *piwi* homologs (Harris and Macdonald, 2001; Kuramochi-Miyagawa et al., 2001), although *piwi* nuclear localization is referred to its protein and not to its mRNA. Nevertheless, *piwi* would not be the first nuclear mRNA reported, although *piwi* dotted staining with WMISH might be due to changes in the staining procedure – this appears unlikely to me, because it is the first example seen among many different transcripts screened.

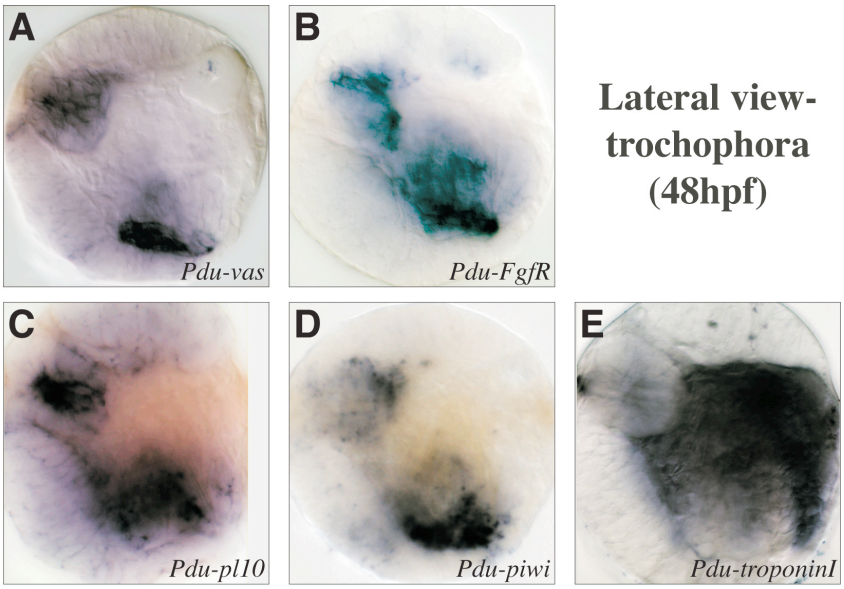


Figure 42. Comparison of genes expressed in the growth zone and stomodaeum

Lateral view of 48hpf *Platynereis* larvae- ventral side facing the left. (A) *Vasa* expression in the middle stomodaeum and in some cells belonging to the growth zone, while (B) *Fgf R*eceptor mRNA is found in precursors of the mesodermal tissue surrounding the stomodaeum, in the mesodermal bans and in the GZ overlapping there with *vasa* and *piwi* positive cells. (C) *Pl10* expression in the middle stomodaeum and in mesodermal bands, and most probably just partially overlapping with *vasa* in the GZ. (D) *Piwi* expression in the upper cells of the forming mesoderm that encloses the stomodaeum and in cells belonging to the GZ. (E) *TroponinI* expression in differentiated mesodermal cell- muscle cells. *Abbreviations*: growth zone, GZ and hours post fertilization, hpf.

3.2.3.1 Conserved function of piwi in the germline

Drosophila piwi is one component of the polar granule, as are the vasa protein and *nanos* mRNA. Both *nanos* and *piwi* genes are essential for germ cell maintenance (Cox et al., 1998; Lin and Spradling, 1997). *Piwi* mutants fail to maintain the GSC. In *Platynereis*, the homologs of these genes, *Pdu-piwi* and *Pdu-vas*, are co-expressed in the growth zone at

48hpf (Figure 42A, D and Figure 43A, B) and 72hpf (Figure 10B and Figure 21F) and co-expressed with *Pdu-nanos* at 72hpf (Figure 24D and Figure 21E). The growth zone is the area from which at 4dpf the four PGCs of this polychaete develop, thus this region is directly linked with germline development. *Piwi* expression in this area highly suggests that the role of *piwi* in germline development is conserved in *Platynereis*.

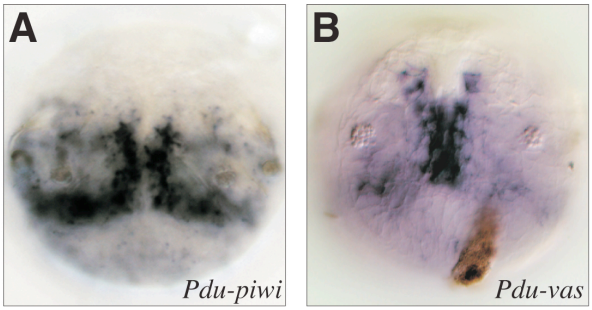


Figure 43. Posterior view of trochophora larvae at 48hpf.

(A) *Piwi* expression as two parallel stripes in the GZ. (B) *Vasa* positive cells forming a "Y" at the GZ. *Abbreviations*: growth zone, GZ and hours post fertilization, hpf.

Conclusions

- *Piwi* mRNA co-expression with *FgfR* mRNA in *Platynereis* implies its role in the Fgf signalling pathway.
- The expression of *piwi* in *Platynereis* suggests a conserved role in germline development as well as in stem cell renewal. Both processes seem to be tightly linked in the trochophora.

3.2.4 Implication of the expression of germline genes in the stomodaeum

The expression pattern of the genes involved in germline formation and screened in my analysis show a strong coincidence between stomodaeum and growth zone formation, and the latter is directly linked to the development of the PGCs. As discussed before, this might be due to the fact that at the stomodaeum at larva stages is highly active, internalizing and changing its shape (discussed above). Nevertheless this activity is not restricted to the stomodaeum. At larval stages, all the tissue is morphologically active although some regions more than others.

A second reason for the dual expression of germline genes in the stomodaeum backs to the ancestral mode of gastrulation of the Bilateria, the amphistomy (Arendt et al., 2001). The blastopore gives rise to both the mouth and anus. The posterior region of the forming gastrula (region from where the germline develop) internalizes, and the relic of genes

expressed in that region is found in both the stomodaeum- the precursor of the mouth, and the growth zone –the precursor of the anus. This is the situation that occurs in the larval stages of *Platynereis*.

Platynereis vasa mRNA is expressed in the stomodaeum (Figure 9C&E and Figure 10B). *Vasa* is not the only gene known to be involved in germline development in other organisms, that in *Platynereis* is expressed both in the germline and in the stomodaeum. Also *Pdu-pl10* (Figure 16D&G), *Pdu-piwi* (Figure 21D&F) and *Pdu-nanos* (Figure 24A&E) are expressed not only in the germline but also in somatic tissue such as the stomodaeum and in the growth zone. In larval stages both regions, the stomodaeum and the growth zone, are the relics of the two regions originated form the blastopore,

Additionally, *Pdu-FgfR* was complementarily expressed with *Pdu-vas* in the stomodaeum, as shown (Figure 44A and B) for a 36hpf trochophora. A similar situation was seen at 72hpf (data not shown). It has been reported that in the mouse embryo neighbouring somatic cells of migratory PGCs express FGF-4 and FGF-8, and they might act as mitogens governing PGC proliferation (Kawase et al., 2004) and *vasa* is expressed in PGCs in mouse (Fujiwara et al., 1994). FGFR-2, one of the two highly expressed receptors, is a major target of FGF-4 in PGCs (Resnick et al., 1998). It might well be that this signalling pathway is also active in *Platynereis*, thus *Pdu-vas* expressing cells might controlled by autocrine and paracrine mechanisms of the FGF signalling pathway. Additionally, *vasa* mRNA is also detected in the growth zone, where the PGCs are formed (discussed previously).

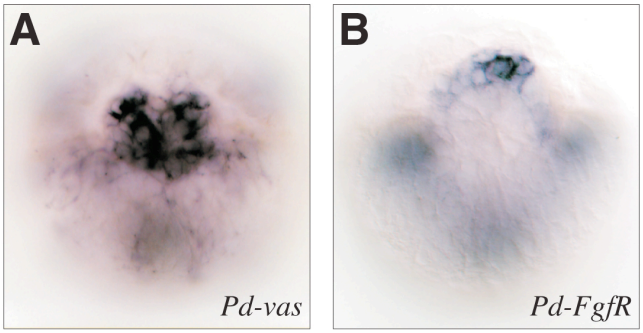


Figure 44. Comparison of *Pdu-vas* mRNA expression to *Pdu-FgfR*

Ventral view of trochophora larval stage [36hpf]: (A) *Pdu-vas* mRNA stomodaeal expression is complemented by *Pdu-FgfR*, seen in (B).

3.3 Conservation in the mechanism in establishing the A/P axis

The second part of this thesis focuses on the analysis of genes, which are known to play a role in germline development and at the same time have a strong link to axial patterning.

The expression pattern of the genes analyzed is complex and not restricted only to one developmental process- in this case, axis formation. In the following, the part of the analysis, beyond germline development, will be discussed.

3.3.1 Nanos, a putative translational repressor of hunchback in the polychaete

Nanos homologs have been reported for several species. In deuterostomes, *nanos* has been found in zebrafish, *Xenopus*, and mouse. In the case of Lophotrochozoans, a leech homolog exists and in this thesis I report its homolog in *Platynereis dumerilii*. For the ecdysozoans *nanos* homologs are present in *C. elegans* and in several insects. Outside the Bilateria homologs have been reported for the cnidarians *Hydra* and *Podocoryne carnea*. *Nanos* seems to be a good marker to study the process of germline formation in animals (see above). Additionally, *nanos* plays an important role in establishing the A/P axis, as it has been described in detail for insects (Lall et al., 2003) and suggested for the hydrozoan *Podocoryne carnea* (Torras et al., 2004).

In early *Drosophila* embryos, nanos protein controls embryonic patterning along the A/P axis. It acts as a posterior determinant (Wang and Lehmann, 1991). Maternal *nanos* mRNA is localized to the posterior pole in the syncytial blastodermal stage. This is achieved by the activity of oskar and vasa proteins (Liang et al., 1994). The function of vasa is to overcome the repressive effect of *nanos* translational control element (Gavis et al., 1996). The zinc finger domain of nanos in turn interacts with *hunchback* mRNA repressing its translation in the posterior of the embryo (Hulskamp et al., 1989; Irish et al., 1989; Tautz, 1988).

Hunchback is a gap gene in *Drosophila* and codes for a zinc-finger type transcription factor that forms an early morphogenetic gradient in the early embryo that is necessary to regulate abdominal gap genes and demarcates the region of the developing head and thoracic segments (Hulskamp et al., 1990; Schulz and Tautz, 1994; Schulz and Tautz, 1995; Struhl et al., 1992). *Hunchback* mRNA requires for its repression the activity of pumilio, which is a sequence-specific RNA-binding protein and translational regulator that recognizes and binds to 'nanos response elements' (NREs) in the 3'UTR of *hunchback* mRNA (Murata and Wharton, 1995; Wharton et al., 1998). The repression of *hunchback* by nanos together with pumilio protein – an RNA binding protein that belongs to the puf

protein family - is essential for abdomen formation of the fly (Hulskamp et al., 1989; Irish et al., 1989; Struhl, 1989; Tautz, 1988).

In order to investigate whether nanos together with pumilio protein represses *hunchback* mRNA and provide evidence for conservation in the mechanism for early embryonic patterning among several species, I cloned and studied the expression pattern of *nanos* mRNA and *hunchback* mRNA in *Platynereis* (see results in chapter 2.1.5 and 2.2.1.2), and performed sequence analysis of one of two putative *pumilio* homologs. The existence of these genes reflects the fact that at least part of the mechanism used in *Drosophila* to establish and pattern the A/P axis has been conserved in *Platynereis*. However, which exact aspects of this mechanism have been conserved?

The example of *Drosophila* – described above – is a good basis to start addressing this question since it is a model system whereas functional studies have been performed. Nevertheless, the mode of development of *Drosophila*, in which the axes of the embryo are already established in the egg, is too different compared to the early embryonic development of *Platynereis*. The cleavage pattern of *Platynereis* represents the canonical unequal spiral cleavage of the Lophotrochozoans (see introduction for more details).

A second example of *nanos* involved in A/P patterning is found in a member of the hydrozoans. In *Podocoryne carnea*, during blastula and gastrula stages *nanos* (*Pcnos1* and *Pcnos2*) transcripts localize to the posterior end of the embryo. During gastrulation, cells at the posterior end move inwards filling the internal cavity. *Nanos* expression remains at the posterior pole, but in cells moving inwards the blastocoel *nanos* mRNA expression ceases. After gastrulation is completed and the bi-layered planula larva is formed, *nanos* expression disappears from the posterior pole (Torras et al., 2004). This is similar to the example seen for *Platynereis nanos* in the prelarva.

At 15hpf (prelarva stage), *Platynereis nanos* mRNA is expressed in a gradient manner with higher levels at the posterior in the mesodermal bands (Figure 45A). Its expression overlaps with *Platynereis hunchback* mRNA at the 1st and 2nd larval segments (Figure 45B). In addition to this, the sequence known as nanos response element –NRE- is found at the 3'UTR of *Platynereis hunchback* (Figure 26B). The localization of both proteins would indicate a role for nanos protein in translational repression of *hunchback* mRNA, and this repression could result in a hunchback protein gradient with higher concentration in the first larval segment. If the repression of *hunchback* mRNA by nanos protein is conserved

at this stage, we would expect hunchback protein in the ventral plate, but excluded or partially excluded from the mesodermal bands. The transcript data presented in this work suggest such a mechanism in *Platynereis dumerilii*, indicating a role for nanos in embryonic pattern formation.

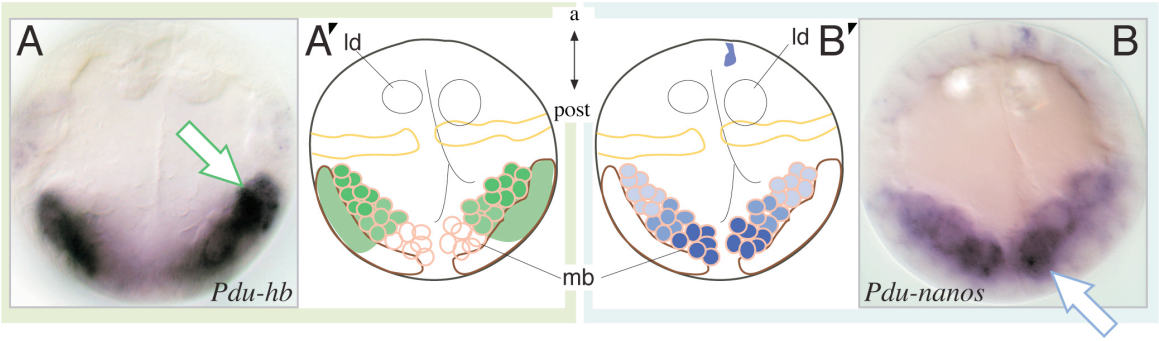


Figure 45. Comparison of *hunchback* and *nanos* expression in the hyposphere

Ventral view of 15hpf larva. (A) *Hunchback* mRNA localization in cells belonging to the mesodermal bands and ectoderm of the region where the 1st and 2nd larval segment will form, while *nanos* mRNA, as seen in (B), is strongly expressed in cells the most posterior cells of the mesodermal band at the region where the 3rd larval segment will later develop, and its expression is decreasing in a gradient manner towards the anterior. *Abbreviations:* lipid droplet, ld; mesodermal bands, mb; posterior, post and anterior, a. Schematics of the correspondent stage are represented with apostrophe (‘). Arrows point at the region where the highest levels of the respective transcripts is detected.

Comparison of the expression pattern of *nanos* to *hunchback* mRNA in the prelarva of *Platynereis*, together with the example seen in insects, and the information about the localization of *nanos* mRNA at the posterior pole during the hydrozoan *Podocoryne* gastrulation, suggest that the ancestor of the protostomes and the cnidarians used nanos to establish the posterior region of the embryo. As far as hunchback role in the process of establishment the anterior segments is concerned, I conclude that its translational repression from the posterior region by nanos protein might be conserved in the protostomes. No hunchback homolog has been yet reported in the deuterostomes.

If we assume that nanos translational repression of *hunchback* mRNA is used to specify the anterior and posterior segments, and if we assume a role for pumilio similar to its role in *Drosophila*, then common mechanisms were operating in representatives of the two major groups of the protostomes: *Platynereis* as a representative of the lophotrochozoans, and *Drosophila* for the ecdysozoans. It would be very interesting to investigate the localization of these proteins and perform gain- and loss of function with these genes.

Conclusions:

1. A conserved mechanism in patterning the A/P axis is suggested by the pattern of expression in the prelarva of *nanos* mRNA when compared to *hunchback* expression pattern.
2. The fact that *nanos* is involved in germline development and might be involved in embryonic patterning repressing *hunchback* in *Platynereis*, further corroborates a link between both of these processes. This link appears to be conserved in insects and lophotrochozoans. Thus, it might be an ancestral feature found in the ancestor of the protostomes.

3.4 Other conserved expression patterns of the analyzed genes

3.4.1 Ancestral expression of nanos mRNA in the mesodermal lineage

Nanos mRNA expression in the mesoderm in *Platynereis* (Figure 23B, F and G) is not the only example of a *nanos* homolog expressed in the mesoderm. The *nanos* homolog in *Podocoryne carnea* is detected in the entocodon, which is a round structure formed at stage 2 (medusa bud formation) by invaginating ectodermal cells (Torras et al., 2004). The entocodon might be considered as a "third germ layer" related to the mesoderm of triploblastic animals (Muller et al., 2003). Furthermore, in leech, *nanos* is expressed in the mesodermal precursor cell and its mRNA remains in the PGCs, which are of mesodermal origin (Kang et al., 2002; Pilon and Weisblat, 1997). The situation in leech is similar to that in *Platynereis* as far as *nanos* expression in the mesoblasts -the mesodermal precursor cells for the polychaetes- and PGCs is concerned (Figure 23B and G and Figure 24D and H). On the other hand, *Platynereis nanos* is not continuously expressed in the mesodermal lineage since at 48hpf the transcripts are neither detected in the growth zone, nor in mesodermal tissue (Figure 24A-C). In conclusion, the expression in the mesoderm of *nanos*-related genes is an ancestral feature, because examples of this feature are reported in cnidarians and protostomes. In *Platynereis*, the germline originates from the mesoderm; this is congruent with expression of *nanos* mRNA in the PGCs in the trochophora (see chapter 3.1.2).

3.4.2 *Nanos* and *pumilio* expression in the brain and nervous system

In *Platynereis*, *nanos* expression was not restricted to the germline. It was also found in somatic tissue – in the brain, neurons and stomodaeum. *Nanos* expression in the brain started with eight *nanos* positive cells at 15hpf (Figure 23C, D and G), at the time when the prototroch forms- the first sign of brain development. In the trochophora, the whole brain the stomodaeal area expressed *nanos* (Figure 24A-C). In addition to this, the midline expressed *nanos* at this stage (Figure 24C). At 72hpf, the expression was restricted to the apical organ, larval eye region, in the relics of the 0 larval segment- which is known to be incorporated in the head-, and in some regions of the stomodaeum (Figure 24D&E). Once the neural plate was formed -at 4dpf-, the transcripts were detected in neurons located in the ventral plate and in the parapodial region (Figure 24F&G). The labelled cells in this region might have been peripheral neurons, since they were located at the surface of the embryo, and some were midline neurons. The latter is congruent with the data reported for *Drosophila*, where peripherial neurons synthesize nanos - together with pumilio - protein (Ye et al., 2004).

Nanos expression in the brain region has also been reported for mouse. During the formation of the hippocampus zygotic *nanos1* was predominantly expressed (Haraguchi et al., 2003). The cnidarian homolog, *Cnnos2*, was specifically expressed in the endoderm of the hypostome where it appears to be involved in head morphogenesis (Mochizuki et al., 2000). These data congruent with the *nanos* expression in the head and stomodaeum of this polychaete and suggests *nanos* implication in head development in the Urbilateria- the putative common ancestor of all metazoans.

The expression of *nanos* in the larval eye region is a feature that might have been acquired in the polychaetes, since it has not yet been reported for other phyla. Although, in mouse *nanos* is expressed in the in the central nervous system (Haraguchi et al., 2003). Moreover, in a similar mechanism as the one used in A/P patterning, *Drosophila nanos* together with *pumilio* regulates dendrite morphogenesis, and without requiring *hunchback*, which is a pivotal player in body patterning (Ye et al., 2004). As I pointed out above, *Platynereis nanos* is also detected in neurons. This might be another conserved feature of *nanos* homologs. In conclusion, *nanos* mRNA expression in brain and neurons in *Platynereis* points to a role of this gene in the CNS development.

3.4.3 Pumilio expression in the nervous system

In the following, I shortly summarize some data reported for *pumilio* homolog in *Drosophila*. Pumilio is an RNA binding protein that interacts with the RNA binding protein nanos to form a translation repression complex essential for antero-posterior body patterning in early *Drosophila* embryogenesis (Johnstone and Lasko, 2001). Pumilio protein has been found to be expressed, and able to repress translation in the neuronal tissue of the *Drosophila* eye (Wharton et al., 1998). A *pumilio* allele known as *pumuckle* was shown to be defective in correct optic nerve path finding (Schmucker et al., 1997) and another allele of it known as *bem* demonstrates that *pumilio* is involved in the maintenance of proper neuronal excitability (Schweers et al., 2002). Thus, several findings implicate *nanos* and *pumilio* in eye development (Wharton et al., 1998), optic nerve development (Schmucker et al., 1997), neuronal excitability (Schweers et al., 2002) and dendrite morphogenesis (Ye et al., 2004).

This thesis reports the existence of at least one *pumilio* homolog; however, no detailed studies have been performed that would answer the question whether the molecular interaction of *nanos* and *pumilio* is persistent in the polychaete. To clarify this issue it will be necessary a) to investigate the expression pattern of *pumilio*, b) to perform gain- and loss of function studies to reveal the dependence of both proteins in the developmental processes, as well as their independent function.

3.4.4 *Hunchback* expression is conserved among the lophotrochozoans

I discussed in chapter 3.3.1 the putative role for *hunchback* in specifying the region of the first two larval segments in this polychaete. In the following, I will focus on other features of *hunchback* in *Platynereis*. Some of these were also reported for the nematode *Caenorhabditis elegans*, the leech *Helobdella triserialis* and the polychaete *Capitella capitata*.

3.4.4.1 *Hunchback* is detected in the ventral plate of annelids

Platynereis hunchback expression during 15-19hpf was not restricted to cells of the mesodermal bands. It also included cells belonging to the ventral plate (Figure 28A-B and D-E). This situation is more obvious when comparing the *hunchback* to *nanos* expression pattern at these stages. As seen in Figure 45A, *hunchback* expression in the hyposphere

reaches the outer layer of the embryo while the expression of *nanos* is localized in the inner embryo, in the mesodermal bands, as shown in Figure 45B. In the leech, *hunchback* was expressed in the germinal plate, which is derived from five bilaterally paired stem cells called teloblasts from which the mesodermal and ectodermal tissue are formed (Savage and Shankland, 1996; Weisblat and Shankland, 1985; Weisblat et al., 1980). In the polychaete, *Capitella capitata*, hunchback protein is synthesized in the micromere-derived surface epithelium that undergoes epiboly and in the large vegetal blastomeres that gradually become internalized (Werbrock et al., 2001). This is similar to the expression pattern observed in leech and in *Platynereis*. Thus, *hunchback* expression in the ventral plate is a conserved feature of annelids.

3.4.4.2 *Platynereis hunchback* is expressed in the precursors of the prostomium and in transient epithelia

The expression of *hunchback* in the brain started at 15hpf in cells at the dorsal side of the brain, extended at 19hpf as half moon like structure covering the future larval eye region. The transcript was detected in the inner ventral brain region as two blocks of cells, one on each side of the embryo. A similar situation has been described for the leech in which cells that occupied the micromere cap - located at the anterior of the embryo - expressed *hunchback* during embryonic stages 4 to 6, which are comparable to *Platynereis* prelarva stages. The cells of the micromere cap give rise to the cephalic region called the prostomium (Savage and Shankland, 1996). Thus, *Platynereis hunchback* expression in the brain resembles the situation reported for the leech. I suggest that *hunchback* positive cells in the brain throughout *Platynereis* early embryonic stages are the precursors of part of the prostomium in this polychaete. In leech, some cells belonging to the micromere cap give rise to a provisional micromere-derived epithelium, which undergoes epiboly covering the embryonic tissue that will give rise to the segmented trunk of the adult (Smith et al., 1996) (Ho and Weisblat, 1987; Iwasa et al., 2000). This provisional epithelium in leech produces hunchback protein during morphogenesis and, like the epithelium of the trochophora, it is a temporary epithelium that will entirely be replaced by the adult cuticle (Savage and Shankland, 1996). In *Platynereis*, the epithelial *hunchback* expression started in a segmental manner at 30hpf. At 72-85hpf, an epithelium expressing *hunchback* had covered the whole embryo (Figure 30A-H). The ectodermal expression reached its highest levels at 5dpf and it was down-regulated in juveniles (Figure 31). Therefore, I conclude that *hunchback* is down-regulated once the larval epithelium is replaced by the presumptive

adult cuticle or "adult" epithelium in *Platynereis*. Although this process has not yet been described in the polychaete, using *hunchback* as a marker for larval epithelium would help to investigate whether this is indeed a common feature in annelids. In the polychaete, *Capitella capitata,* an epithelial expression of *hunchback* has been reported in the leading edge of the endoderm undergoing epiboly and in the epithelium of the pygidial and prostomium area (Werbrock et al., 2001). The nematode homolog for *hunchback* (*hbl-1*) was expressed in epithelial precursor cells before organogenesis. RNAi experiments showed that embryos without functional hunchback failed to elongate (Fay et al., 1999). Thus, examples in four species (one for the leeches: *Helobdella triserialis*, two for the polychaetes: *Capitella capitata* and *Platynereis dumerilii*, and one for the nematodes: *Caenorhabditis elegans*) report *hunchback* expression in temporary epithelium at the time when morphogenetic events are taking place, although a direct link has only been demonstrated in *Caenorhabditis elegans.*

3.4.4.3 *Hunchback* expression in the stomatoblasts

Early studies on annelid development described stomodaeal precursor cells (called stomatoblasts for polychaetes) first to form at the surface and then to internalize below the surface epithelium to form a tube in front of the presumptive midgut at the ventral midline (Anderson, 1973). A description of *Nereis* development reported the stomatoblasts ($2c^2$ and $2a^2$ blastomeres) left and right of the embryo to converge together with other cells to the central point, where the longitudinal slit-like blastopore lips fuse along the later ventral midline (Arendt et al., 2001; Wilson, 1892). Wilson (1892) described that the stomatoblasts divide radially while the larval foregut is forming, and are drawn together in an arc like structure behind the stomodaeum opening. *Hunchback* mRNA expression in the stomatoblasts at 22-24hpf in *Platynereis*, demonstrated that not two, but four stomatoblasts exist in *Platynereis*. These *hunchback* positive cells divided enclosing the mouth at 24hpf. At 30hpf there were no *hunchback* positive cells in the stomodaeal area. *Hunchback* expression in stomodaeal cells has been reported in another polychaete, *Capitella capitata.* In *Capitella capitata* at stage 10 (=prototroch formation) hunchback protein containing cells were found at the surface of the stomodaeum. These cells appeared to become internalized within a few hours (~4 hours) and continued containing hunchback protein (Werbrock et al., 2001). It was also described for the leech that hunchback protein containing cells formed a ring of superficial epithelial cells that correlated with the position

of the prospective mouth at stage 8 of development (Iwasa et al., 2000; Savage and Shankland, 1996). Thus, data reported for *hunchback* homologs in the leech and in two polychaetes, suggest that *hunchback* expression in the stomatoblasts is a conserved feature in the annelids. The function of this gene in the development of the stomodaeum remains to be elucidated.

3.4.4.4 Up-regulation of *hunchback* mRNA in fully matured worms

Platynereis oocytes did not contain *hunchback* transcripts, although their levels of expression in fully matured worms were obviously up-regulated when compared to worms entering epitoky (Figure 25), implying a role of hunchback during the development of this stage. The up-regulation of *hunchback* transcript might be due to one of two reasons: first, *Platynereis* undergoes several morphological changes during epitoky, which imply structural reorganizations of musculature, parapodia, chaeta, eyes and some other structures of the benthic worms (Hauenschild and Fischer, 1969). These morphological changes allow this polychaete to swim to the surface for the 'nuptial dance'. Thus, *hunchback* might be involved in morphogenesis during maturation. The second reason is that *hunchback* mRNA is translated in the female and the protein is stored in the oocytes, and the maternal contribution in this case would be the hunchback protein. This is congruent with the information known of *hunchback* homologs in other species, where its mRNA is maternally contributed to the oocyte (Fay et al., 1999; Savage and Shankland, 1996; Tautz et al., 1987; Werbrock et al., 2001). A feature in which *Platynereis hunchback* differed from the rest of the known homologs were the high levels of expression in fully matured males (Figure 25), which was at least 4 times higher than in females. To unravel *hunchback* role during gametogenesis, it would be necessary to precisely localize its transcript at this stage, both in females and males.

Drosophila hunchback is involved in patterning of the CNS and the peripheral nervous system (Rohr et al., 1999; Wolff et al., 1998) (Kambadur et al., 1998). The homologs for *hunchback* in the leech, nematode and the polychaete *C. capitata* are also expressed in the CNS. The results obtained with WMISH do not show detectable levels of *hunchback* mRNA in the CNS of *Platynereis* at late developmental stages, besides its early expression in the larval eye region at prelarva stage (Figure 28H). Therefore, I conclude that the late *hunchback* expression in the CNS is not conserved in *Platynereis*.

Conclusions:

- *Platynereis* nanos protein might interact with *hunchback* mRNA by binding to its conserved NRE. This interaction was discussed in detailed in chapter 3.3.1.
- *Platynereis hunchback* is expressed in the ventral plate and in the mesodermal bands in a similar manner as its homologs in leech and in the polychaete *Capitella capitata*.
- *Hunchback* possibly contributes to the formation of the stomodaeum, probably by promoting its internalization.
- *Hunchback* mRNA expression suggests its involvement in the development of a temporary epithelium at the time when the trochophora metamorphoses into a young worm.
- In *Platynereis*, hunchback plays a role during the stage of gametogenesis, and especially in males. This might due to a) changes in morphology during epitoky, and b) storage of hunchback protein in the gametes, most probably in oocytes.

4 MATERIALS AND METHODS

4.1 Technical equipment

Eppendorf centrifuge 5417C, rotor F45-3011 and Sorvall rotor H4000

Sectioning system:

Vibratome (vibrating blade microtome): Leica VT1000S, manufactured by Leica Microsystems Nussloch GmbH, Germany. Microtome: Reichert-Jung, Biocut 2030 with a Leica Blade (16cm/c).

Injector Blades, Stainless Steel Teflon-coated, dispenser (pka/20), PLANO, Cat. #121-4.

Tissue Specimen Mounting (plastic) Blocks, PLANO, Cat. # 10076 (pkg/3).

Microscopes: Zeiss Axiophot and Zeiss Axiovert 200.

Objective: Zeiss 5x, 10x, 20x, 40x and 63x air objectives.

Camera: Zeiss AxioCam HRc, software: AxioVision 3.1 and Olympus, Analysis Image Processing.

Mesh for collecting *Platynereis d.* embryos: nylon-sieve tissue NITEX, Maschenweite Type 03-100/44 (Gebr. Stallmann, Suentelstrasse 82,25462 Rellingen b. Hamburg) and NY15 HC (HYDRO-BIOS KIEL)

Computer programs used for analyzing data: BioDoc Analyse 1.0 software (Biometra), ClustalX, DNA Strider 1.3, SeqMan, MegAlign, and EditSeq.

4.2 Worm culture

Platynereis dumerilii material was obtained from an inbred culture maintained at the University of Heidelberg and at EMBL. Both are daughter cultures from the culture bred at the University of Mainz since the sixties. These worms originated from the Gulf of Naples. During the last 20 years two incrosses from other regions has been added.

Worms are bred at 18 °C and according to the standard protocol, for more information see http://www.uni-giessen.de/%7Egf1307/breeding.htm and (Dorresteijn et al., 1993). Two to 29-cell stages corresponded to 2.10 hours post fertilization (hpf) up to 4.50hpf in the culture kept at 18°C at EMBL in Heidelberg. This time scale does not conform to the one reported by Adriaan Dorresteijn (Dorresteijn et al., 1993), when 29-cell stage was reached

at 4.30hpf at 18°C. The difference was most likely due to random oscillations in the temperature.

4.3 Standard cloning vectors and strains

PCR products were directly cloned using the TopoTA kit (Invitrogen). The bacterial strain used for amplification of the selected plasmids was E.coli XL1 blue. Phage cDNA libraries used for cloning were λ-Zap with ZAP^{TM}XR-Vector. Clones from the *Platynereis d*. EST BAC library were incorporated into E.coli DH10B.

4.4 Chemicals and Solutions

This chapter aims at giving a description of general buffers and solutions, since special buffers and reagents are mentioned along with the respective method. Most of the chemicals were purchased from Applichem, Merck, Roth and Sigma, otherwise the provider is noted between brackets along the correspondent product. If working with RNA was intended, solutions were made with DEPC-treated water or sterile filtered.

20xSSPE pH 7.4	3 M NaCl; 0.2 M NaH2PO4; 0.02 M EDTA
TE	10 mM Tris/HCl pH 7.4;1mM EDTA pH 8.0
10xTBE	890 mM Tris; 890 mM boric acid; 20 mM EDTA pH 8.0
50xTAE	242 mg Tris base; 57.1 ml acetic acid; 100ml 0.5M EDTA; and add ddH2O to 1 litre and adjust pH to 8.5.
TBS-T	0.14M NaCl, 2.5mM KCl, 25mM Tris pH7.5, 0.1% Tween 20
1xTNT	0.15M NaCl, 0.1M Tris pH7.5, 0.1% Tween 20
1xTNB	TNT containing 1%NEN TSA blocking reagent, when preparing, heat mixture to app. 55°C and stir to dissolve the blocking reagent more quickly, cool down before use!
LB medium and LB agar	According to the protocol in (Sambrook et al., 1989). Ampecillin concentration of 50 μg/ml
MEMPFA-T	0.1M MOPS pH 7.4; 2mM EGTA; 1mM MgSO4; 4% PFA; 0.1% Tween 20
20xSSC	3M NaCl (175.32g/l) and 0.3M Trinatrium citrate (88.23g/l)
SSCT	SSC, containing 0.1% of Tween-20

10xPBS	70g NaCl; 62.4g Na2HPO4.2H2O; 3.4g KH2PO4 pH 7.4
PBT	PBS containing 0.1% BSA and 0.1% Triton X-100
50xDenhardt's reagent	1% BSA; 1% Ficoll 400; 1% Polyvinylpyrrolidon
PTW	PBS containing 0.1% Tween 20
AP buffer	100mM NaCl; 5mM MgCl2; 100mM Tris pH 9.5; 0.1% Tween 20
Hyb-mix	50% formamide, 5xSSC, 50µg/ml Heparin, 5mg/ml Torula-RNA and 0.1% Tween-20
Denaturing solution	0.5M NaOH/ 1.5M NaCl
Neutralizing solution	1.5M NaCl/ 0.5M Tris pH 7.5

4.5 Antibodies

The polyclonal Formosa antibodies (Chang et al., 2002)- raised against the Grasshopper vasa protein (kind gift of M. Akam) - were used with a dilution of 1:500 on Western blots. The protein extraction was done according to (Ferby et al., 1999). 50µg were loaded per lane in a 12% acrylamide gel and the membrane was treated according to (Schmitt and Nebreda, 2002). Immunocytochemistry was performed with these antibodies on different developmental stages (8hpf to 72hpf). A 1:30 dilution was used to label 48hpf larvae, giving a clear signal that was not observed in the negative control. ABC kit of Vectastain was used to develop the signal.

4.6 Whole mount in situ hybridization (WMISH)

All steps were performed at room temperature unless otherwise noted. Embryos were collected with a nylon-mesh. This net (or mesh) let pass small particles and algae, keeping embryos or young worms on the net.

4.6.1 RNA probe synthesis for WMISH

The plasmid containing the desired insert (in my case in pCR II-TOPO vector) was linearized, leaving one promoter site active to transcribe an anti- or a sense- RNA probe.

The starting material was 10µg of plasmid. It was digested with 2.5µl of restriction enzyme adding 5µl buffer and H2O to a total volume of 50µl (digestion time: 2hr to a maximum of 4hr, but never overnight).

Keep in mind: in case of digesting a mini prep, it should have been eluted in H2O and not in TE or any other buffer, to avoid interference with enzyme activity. In case of using a plasmid from maxi or midi prep, a small amount of TE would not affect the activity of the enzyme.

Usually the gene of interest was cloned in two parts - 5' and 3' race. To this end, I synthesized both ends in a different reaction and added the same amount of probe to the samples.

Linearized digest was purified with Quiaquick nucleotide removal kit (Qiagen):

- Add 10 volume of Buffer PN (500µl PN to 50µl digest), apply to Quiaquick column and spin 1' 6000rpm, discard the flow through
- Add 750µl PE buffer, spin 1' 6000rpm; discard the flow through
- Put the column back in the tube and spin it for 1' 13000rpm
- Elute DNA in 2x25µl H2O, spin 1' 13000 rpm
- Load 1µl of purified digest on a 1.5% agarose gel to check for traces of undigested plasmid

4.6.1.1 RNA probe preparation

Compounds were added in the following order:

Linearized template 5µl (1µg to 300ng)

100mM DTT	2µl
NTP-Mix	1.3µl
10mM Dig-UTP	0.7µl
RNase inhibitor (Fermentas, 40U/µl)	0.5µl
10xTranscription Buffer	2µl
H_2O	7.5µl
RNA polymerase (SP6 or T7)	1µl

NTP-Mix:

GTP 15.4 mM

CTP 15.4 mM

They were mixed and incubated for 2,5 hr at 37°C, then 1µl DNase I (Roche, 10U/µl) was added and incubated 15min at 37°C. The reaction was purified with RNeasy kit (Qiagen) according to the manufacture's protocol, and eluted twice with 25µl RNase-free water. 2µl of the reaction was separated in an extra RNase-free tube and combined with 3µl of RNA loading dye; later it was cook for 4 min at 80°C and loaded into a 1.5% agarose gel in TAE. A DNA Mass Ruler was used in case of lacking an RNA Mass Ruler, in this case the size of the band was just roughly estimated. The rest of the reaction was stored in 150µl of Hyb-mix at -20°C.

4.6.2 WMISH for 0hpf to younger than 15hpf

HB (hybridization buffer)

750mM NaCl, 75 mM Na citrate pH 7.0, 50% Formamide, 100 µg/ml Torula RNA (Fulka), 1.5% blocking reagent (Roche), 5 mM EDTA, 0.1% Tween-20

MaNaT

100 mM maleic acid/150 mM NaCl pH 7.5, containing 0.1% Tween-20

BB (Blocking Buffer)

MaNaT solution with 1% of blocking reagent, the blocking reagent should be warmed up to dissolve and let it cooled down before applying to the samples.

APB (Alkaline phosphatase buffer)

100mM Tris, pH 9.5, 50mM $MgCl_2$, 100 mM NaCl

Dabco-glycerol

25mg Dabco in 1 ml in PBS and 9 ml of glycerol

The WMISH protocol was established for older stages from *Platynereis dumerilii* (see chapter 4.6.3). Neither the original protocol nor any modifications made for older stages worked in younger stages, therefore a new protocol was needed for younger stages (means younger than 15hpf). After studying some WMISH protocols used in other organisms I chose the following protocol for younger stages based on the in situ hybridization protocol used for *Patella vulgata* (Lartillot et al., 2002b; Lespinet et al., 2002).

Embryos were de-jellied by a brief treatment in Millipore-filtered sea water (MPFSW) acidified with HCl to ph 3.9 (for one litre of natural sea water (NSW) add 12 drops of 32%

HCl) and then washed in normal MPFSW few times. Embryos were then treated according as follows:

- Fix embryos for one hr with MEMPFA-T on a rotating wheel
- Dehydrate them progressively with 5 min washes with a gradual series of methanol/MEMPFA-T (25/75, 50/50, 75/25, 100/0) followed by a 15 min wash with 100% MeOH. At this point embryos should be stored at –20ºC for at least 2 hr (better overnight) or until further use.
- Progressively re-hydrate them in TBS-T by 5 min successive washes in methanol/TBS-T (75/25, 50/50, 25/75, 0/100)
- Digest with Proteinase K (50ng/ml; Roche Molecular Biochemicals) in TBS-T for 20 min at 37ºC
- Incubate in the following order,
  - 0.1M triethanolamine (TEA, Merck) pH 7.0-8.0
  - TEA with 2.5 µl acetic anhydride/ml (Baker Chemicals)
  - TEA with 5.0 µl acetic anhydride/ml
- Wash 2x for 5 min in TBS-T
- Re-fix in MEMPFA-T for 20min
- Wash 5x5min in TBS-T
- Wash for 10 min in 500 µl 50/50 TBS-T/HB
- Pre-hybridize in 100 µl HB in an Eppendorf tube for 6hr at 68ºC
- Add probe to 1µg/ml and hybridize overnight at 68ºC
- Remove the probe by washes at 68ºC in 50/50 HB/2xSSC for 20 min
  - 25/75 HB/2xSSC for 20 min
  - 2xSSC for 30 min
  - 2x 0.2xSSC with 0.3%CHAPS (Sigma) for 30 min
- Wash 2x in MaNaT for 10 min at RT

- Block for 1 hr with BB
- Add Anti-Dig antibody (coupled with alkaline phosphatase at a 1:200 dilution) for 1 hr in fresh BB solution
- Wash in MaNaT for 2x5min and 6x15min
- Wash embryos with APB
- Stain in a fresh tube with APB containing: PVA (10% polyvinyl alcohol, 100kDa), 3.5 µl NBT (nitro blue tetrazolium; 75mg/ml, Roche) and 3.5 µl BCIP (5-bromo-4-chloro-3-indolyphosphate; 50mg/ml; Roche) per ml buffer at 37ºC
- Stop staining reaction by washing 2x with TBS-T
- Incubate with Hoechst (5 µg/ml in TBS-T) for 10 min at RT to stain the nucleus (from now on, keep in dark)
- Re-fix with MEMPFA-T for 20 min
- Wash 2x with TBS-T
- Transfer the embryos in Dabco-glycerol, store at 4ºC in dark.

Samples were then ready for microscopy.

4.6.3 WMISH for older embryos and adult worms

Sense and anti-sense probes were synthesized from linearized plasmids and T7/ SP6 RNA polymerase and labelled with digoxigenin-12-UTP. Embryos and adult worms were fixed in 4% PFA / 2xPTW for 2 hrs. The protocol for worms of 15hpf and older was modified after (Loosli et al., 1998). Worms older than 1 month were kept without food during 1 to 2 weeks, procuring them to have a relatively empty gut because food in the gut was a source of background staining.

All steps were preformed at room temperature unless indicated otherwise. Larval stages and adult worms were fixed in 4% paraformaldehyde /2X PBS-Tween (PFA/2XPTW) for 2 hours and then stored at –20 °C in 100% methanol (MeOH). Embryos were progressively re-hydrated in PTW by 5 min successive washes in MeOH/PTW (75/25, 50/50, 25/75, 2x 0/100). Then they were treated with Proteinase K (100µg/ml, Roche) in PTW for 1 to 2 min depending on the stage of development:

15-24hpf	30 sec
24-72hpf	1 min
72hpf-5days	2 min
older than 5days	2 1/2 to 3 min

After that they were rinsed twice in freshly prepared glycin (2mg/ml) in PTW. They were post-fixed in 4%PFA/PTW for 20 min and then washed 5 times for 5 min in PTW. Hybridization steps were carried out at 65 °C. Embryos were prehybridized for 1 hr in hybridization buffer (HB), then probe was added (10 ng/µl) and left hybridizing overnight. The probe was removed by washes at 65 °C in 50% formamide/ 2xSSCT for 30 min, twice in 2xSSCT for 15 min and twice in 0.2xSSCT for 30 min. Embryos were blocked for 1 hr with 5% sheep serum in PTW, and then incubated for 1 hour in sheep serum (2.5%)/PTW blocked anti-Dig –AP Fab fragments. Wash the embryos 6 times in PTw for 10 min, then equilibrate them in APB by two rinses of 5 min. The staining was induced by adding 4.5 µl NBT (100mg/ml) and 3.5 µl BCIP (50mg/ml) per ml of buffer and was stopped by rinsing the samples first with APB and then twice with PTw. Larvae were then post fixed in 4% PFA/PTW for 1 hour, rinsed twice with PTW and then transferred to 87% glycerol. Embryos were mounted in glycerol and pictures were taken under the Nomarsky optics using a Zeiss Axiophot and/or Zeiss Axiovert 200.

4.7 Isolation of total RNA

For extraction of total RNA from worms older than 1.5 or 2 months and longer than 20-25 segments, a single worm was used, otherwise one or two batches were collected into a cut Falcon tube where one opening was sealed or blocked with a nylon mesh. This mesh would allow sea water and small particles to pass through but not the worms of any stage. In this way worms were collected in a small volume of water and then transferred to 2ml eppendorf tube, which was cut open at the base of the tube. The lid of this tube was perforated and a small nylon mesh could be placed between the lid and the tube. In this way the worms were collected in an even smaller volume of water. After most of the sea water dripped out of the tube, the worms together with the net were placed into a 2 ml eppendorf tube, and shock frozen in liquid nitrogen or transferred immediately into a -

70°C freezer. Total RNA was extracted with TRIzol®Reagent (Invitrogen, #15596-026) and according to the manufacturer's protocol. 1ml of Trizol was applied to each net. The worms were homogenized using a plastic stick, which matched exactly with the inner space of a 2ml eppendorf tube. The results of the extraction were quantified with photo-spectrometry, the quality of the RNA was tested by gel electrophoresis and further checked with RT-PCR using actin as a standard.

4.8 Synthesis of single stranded cDNA

Total RNA was used as a template to synthesize single stranded cDNA for degenerated PCR and extension of fragments to the C-terminus (3'race PCR). For this case, SuperScriptII Reverse transcriptase (Gibco) was used according to the manufacturer's protocol. cDNA libraries used to set up semi-quantitative RT-PCR were done with Revert Aid First Strand Synthesis Kit (Fermentas, #EP0441) as described in the manual of the manufacturer, starting with 5μg of total RNA.

With the purpose of extending existing fragments towards the N-Terminus of the gene (5'race PCR) the SMART RACE cDNA Amplification Kit (Clontech, #K1811-1) was used following the producer's protocol.

4.9 Northern Blots

4.9.1 Synthesis of the RNA probe

The synthesis of the probe was done according to the protocol used to synthesize the probe for WMISH (see chapter 4.6.1). Good results were obtained with 7 μl of probe in 5 ml of hybridization solution.

4.9.2 Preparation of the formaldehyde-gel

Running buffer

50 ml of 10 x MOPS

10 ml of 37 % formaldehyde

440 ml of DEPC-treated water

Preparation of the formaldehyde-gel (1 %)

The bottle was rinsed with DEPC- treated water; the gel plate, comb and chamber were soaked in DEPC-treated water. 0.6g agarose, 5ml 10xMOPS and 45 ml DEPC-treated water were added in the bottle. It was slowly heated until the small agarose particles dissolved, cooled down to 60-70 °C and 900 µl of 37 % formaldehyde was added. After this it was mixed and poured.

Loading the gel

8 µg of total RNA per sample was loaded, and RNA loading dye (containing EtBr, Fermentas) was used. The gel ran at 100 mV, a picture of the gel was taken before building the blot procuring a minimum of exposure to UV light.

4.9.3 Building the blot

The blot was put together according to the drawing depicted in Figure 46. This steps were followed: the first two Whatmann papers were rinsed shortly in 20x SSC, then placed on the chamber followed by the gel with the bottom side facing up. The Hybond N membrane was rinsed shortly in 20x SSC, laid above the gel taking care that no bubble remained trapped between the two layers. The last 3 layers of Whatmann paper were soaked in 2x SSC and put onto the membrane. On the top of this, many layers of paper towels facilitated the transfer of buffer, and at the same time the transfer of the RNA to the nylon membrane. A glass plate and a filled 50ml Falcon tube were used as weight to equilibrate the set up. The transfer took place overnight.

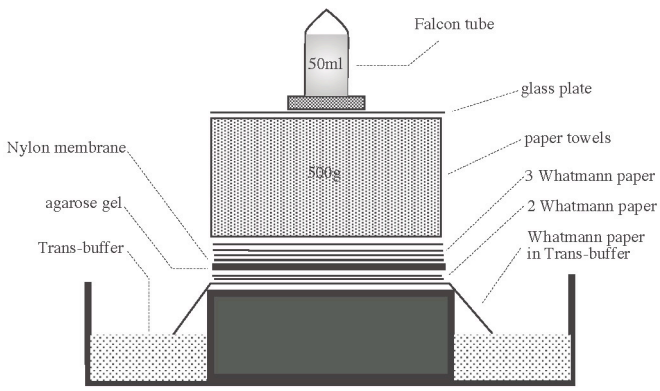


Figure 46. Drawing of Northern Blot Transfer

A chamber was filled up with transferring buffer and paper towels absorbed this buffer. In this manner RNA samples were transferred to the Hybond N membrane.

4.9.4 Hybridizing and developing of the blot

Pre-hybridization solution

5x SSPE

1x Denhardt's reagent

0.7% (w/v) SDS; 50% (v/v) formamide

125 µg/ml salmon sperm DNA (add after denaturing it for 10min at 95°C)

Hybridization solution

90% Pre-hybridization solution

10% (v/v) Dextransulfate

and denatured DIG-labelled RNA probe (1.4µl/ml of Hybridization sol.)

After transferring the RNA from the agarose gel to the Hybond N membrane, the loaded slots were marked with a fine pencil and the membrane was washed in 6xSSC for 5 min. The membrane was then kept between two Whatmann paper layers and let dry. After the membrane was completely dried, it was baked for 30 min at 120°C. The RNA markers were cut out and their bands were made visible by soaking the markers for a maximum of 10min in 0.2%-Methylenblue/0.5M sodium acetate pH 5.2. The membrane was placed in a Falcon tube and rinsed once with 5xSSPE, later prehybridized for 2hrs and hybridized overnight. Both steps took place at 63°C in a hybridization oven. The salmon sperm was denatured at 95°C for 10min before being added. For the hybridization step the DIG-labelled RNA probe was also denatured with the same parameters mentioned before. The membrane was then washed twice at room temperature in 2xSSC containing 0.1% SDS, consecutively washed twice at 65°C in 0.1xSSC containing 0.1% SDS. The membrane was next blocked in PBT for 30min. AP-anti-DIG antibody was then added with a 1:3500 dilution in PBT. Tree times consecutive PBT washes of 10min each followed and the membrane was after that transferred to AP buffer. The staining reaction was started with NBT (0.35 mg/ml, Roche) and BCIP (0.175 mg/ml, Roche) in AP buffer, and after 20min of staining the reaction was stopped with several washes of DEPC-treated sterile water.

4.10 Cloning procedures

4.10.1 Cloning of novel genes with degenerated primers

The cloning of novel gene fragments for the polychaete *Platynereis dumerilii* was achieved by designing degenerated primers (Buck and Axel, 1991) in conserved regions, based on the AA sequence alignment of different homologs of the gene of interest. The parameters of the primers were checked using the Oligo 6.44 program for Macintosh. All primers were synthesized at MWG Biotech AG. These primers were used in a degenerated PCR using as template phage- or single stranded cDNA libraries. 10µl of the PCR product were analyzed on agarose gel. The band obtained with the expected size (compared to the known homologs in other species) was gel eluted with GFX™ Gel Band Purification Kit (Amersham Pharmacia Biotech) and cloned into the pCR II®-TOPO vector using the TopoTA kit (Invitrogen). 30 to 60 colonies were picked and mini-prepped using Plasmid Purification Kit (QIAGEN). The resulting plasmids were separately digested with EcoRI and HinfI. Plasmids with the correct insert size (according to the AA sequence alignment), but different HinfI digest patterns were sequenced, some by the Genomics Core Facility at EMBL, Heidelberg, and some by Medigenomix, Germany.

4.10.2 Extensions of existing fragments

Based on existing fragments, specific primers were designed using Oligo 6.44 program. The location of these primers was chosen in such way that after extensions of the gene to the N- or C-Terminus (5' or 3' race) an overlap of 100 to 150bp between the fragment and the race would still exist. Race PCR were preformed using a specific primer for the gene of interest and a specific primer for the library used:

library	**specific library primer**	**PCR program**
ss cDNA	Race Ada	Race Ada PCR
smart cDNA	UPM mix	Smart Race PCR
λ-Zap	T7 or T3	Race PCR

A nested PCR was applied to re-amplified extended fragments, using the product of the first PCR as a template, gene specific primers and library specific primers. All the products

were subjected to gel electrophoresis, then the gel was Southern-blotted and hybridized with high stringent conditions, using the existing fragment of the gene as a template to synthesize a radioactive probe. Bands that gave a positive signal were then cut out from the gel, gel eluted and subcloned using the TopoTA Cloning Kit (Invitrogen). The steps that followed were the same as the ones described above.

4.11 Polymerase chain reaction (PCR)

Different programs and mixtures were utilized depending on the purpose for setting up the reaction. The extension time for each reaction was calculated for 1000bps/min.

4.11.1 Templates and mixtures

λ-Zap cDNA libraries (gift form C. Heimann and A. Dorresteijn)

Platynereis dumerilii stages: 24hpf-Trochophora and 48hpf-Metatrochophora, ZAP^{TM}XR-Vector (STRATEGENE), amplified to 7.0 *10^{10} and 1.5*10^{11} pfu/ml respectively. Race PCR mixture was assembled according to the following parameters:

4 μl of λ-Zap library

5 μl of dNTPs (5mM)

1.5 μl of specific primer (100mM)

1.5 μl of T3/T7 primer (100mM)

5 μl of PCR Buffer (10x, Qiagen)

0.2 μl of Taq DNA polymerase (Qiagen)

32.8 μl of deionised water

Smart Race cDNA libraries

cDNA libraries of different stages of *Platynereis d.* development were made using Smart Race cDNA libraries amplification Kit (Clontech #K1811-1). Smart Race PCR was assembled according to the followings:

2-3 μl of Smart Race cDNA library

5 μl of dNTPs (5mM)

1 μl of specific primer (100mM)

2.5 μl of UPM primer mix (see kit)

5 μl of PCR Buffer (10x,Qiagen)

0.2 μl of Taq DNA polymerase (Qiagen)

34.3 μl of deionised water

single stranded cDNA libraries (ss cDNA)

Single stranded cDNA libraries of different stages of *Platynereis d*. development were used for extension of existing gene fragments to its C-terminus (3' race PCR) and to obtain approximate estimation of levels of expression of transcripts (semi quantitative RT-PCR). Mixture for Race Ada PCR was assembled according to the following parameters:

1 μl of ss. cDNA

4 μl of dNTPs (5mM)

1 μl of specific forwarded primer (100mM)

1μl of T17Race Ada primer (100mM)

1μl of Race Ada primer (100mM)

5 μl of PCR Buffer (10x, Qiagen)

0.2 μl of Taq DNA polymerase (Qiagen)

36.8 μl of deionised water

The amount of library used for each RT-PCR was standardised based on the transcript levels of actin and the help of the forwarded primer: GGAGACGATGCTCCCAGAGCT (actin_fw2) and reversed primer: AACTCTCAATTCATTGTAGAAGGTGT (actin_rev2) specific for *Platynereis d.*, the mixture used was the following:

0.2-0.4 μl of ss. cDNA

1.2 μl of dNTPs (2mM)

1 μl of specific forwarded primer (10mM)

1 μl of specific reversed primer (10mM)

1.6 μl of $MgCl_2$ (25mM)

2 μl of PCR Buffer (10x, Fermentas)

0.2 μl of Taq DNA polymerase (5U/μl, Fermentas)

deionised water to a total of 20μl

Mixture for cloning novel genes

When a novel gene was cloned using degenerated primer then any of the upper described libraries was used as a starting template, and the following mixture was assembled for degenerated PCR:

2 μl of cDNA library

5 μl of dNTPs (5mM)

1 μl of degenerated forwarded primer (100mM)

1μl of degenerated reversed primer (100mM)

5 μl of PCR Buffer (10x, Qiagen)

0.2 μl of Taq DNA polymerase (Qiagen)

35.8 μl of deionised water

Mixture for nested PCR

After obtaining the products of the first PCR, usually a second PCR was made using a nested primer to amplified or/and corroborate the results of the first PCR. In this case the following mixture was used:

1 μl of the first PCR product

5 μl of dNTPs (5mM)

1 μl of specific, or degenerated forwarded primer (100mM)

1 μl of specific primer, or reversed degenerated primer (100mM)

5 μl of PCR Buffer (10x, Qiagen)

0.2 μl of Taq polymerase (Qiagen)

36.8 μl of deionised water

4.11.2 programs

Each PCR began regularly with a 'Hot start', the x time value giving to each program varied according to the number of samples for each PCR.

Race PCR and Smart Race PCR

1. x' 94°C (Hot start)
2. 30" 94°C
3. 1' 60°C
4. 4' 72°C go to 2 34x
5. 10' 72°C
6. End

Race Ada PCR

1. x' 94 °C (Hot start)
2. 1' 94 °C
3. 1' 60 °C
4. 4' 72°C go to 2 10x

Add 1µl of oligo T17Race Ada

5. 1' 94 °C
6. 1' 35 °C
7. 4' 72°C go to 5 3x

Add 1µl of Race Ada oligo

8. 1' 94 °C
9. 1' 45 °C
10. 4' 72°C go to 8 20x
11. 10' 72°C
12. End

Degenerated PCR

1. 2’ 94°C (Hot start)
2. 1’ 94 °C
3. 2’ (Tm-5) °C
4. 4’ 72°C go to 2 5x
5. 1’ 94 °C
6. 2’ Tm °C
7. 4’ 72°C go to 5 35x
8. 10’ 72°C
9. End

Tm: Optimal melting temperature of degenerated forwarded and reversed primer pair (value calculated with Oligo

Nested PCR

1. x’ 94°C (Hot start)
2. 30” 94°C
3. 1’ 60°C
4. 5’ 72°C go to 2 34x
5. 10’ 72°C
6. End

Semi quantitative RT-PCR

1. x’ 96°C (Hot start)
2. 1’ 96°C
3. 45” 60°C
4. 45” 72°C go to 2 29x
5. 10’ 72°C
6. End

4.11.3 primers

The following specific primers were used for cloning novel genes, elongation of known genes, sequencing or/and RT-PCR.

Table 1. General primers

primer name	sequence (5' to 3' end)
Race Ada	ACTCGAGTCGACATCG
T17Race Ada	GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT
T3	ATTAACCCTCACTAAAG
T7	CATTATGCTGAGTGATATCCCG
UPM Long	CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGT
UPM Short	CTAATACGACTCACTATAGGGC
SP6	ATTTAGGTGACACTATAG

Table 2. Primers designed to target specific genes

target gene	primer name	DNA sequence (5'-3')
Vasa	LO1	CKNCCDATICKRTGIACRTAITCRTCDAT
	LO2	SWNGGCATRTCRTARTTDATIACRTG
	LO3	TGYTTNACRTCISWRCAIGCICCICCIAC
	LO4	CCAAGCTTNARCATNCKRTCNGCYTCRTC
	UP1	AAYTTYWSIAARTAYGAYRAYATHCCIGT
	UP1.5	GAYYTNATGGCITGYGCICARAC
	UP1.7	CCGGATCCATGGCNTGYGCNCARACNG
	UP1.8	CCCGGATCCCCNACNMGNGARYTNRYNNWNCARATH
	UP3	GARGCNGAYMGIATGYTIGAYATGGGITT
	UP4	GCNACITTYCCIGARGARATHCA
	UP5	GTNGGNRTIGTIGGIGGIGCITG
	vasa_seq_up	ACTTTGACAAATACGAGT
	vasa_seq_lo	GACAACGTGGGCGCCCTT
	vasa-race2_up1	GCGCAGTGCTGATTTCCTTGCTG

	vasa-race2_up2	CCCAAGAACAGTACCCTGCCACA
	vasa-race2_lo1	CCGGCCTGTGATGGACTGGAACA
	vasa-race2_lo2	AGCCGGGGGAACATAGATCTCAG
	race_vasa_up1	TGTGTTTGTGACTGTCGGACGAG
	race_vasa_lo1	GCCACTGGGGAGATCGTA
	race_vasa_up2	GTGCCAACTCCGACATTACTC
	race_vasa_lo2	GCAGCCACTGAGGTAGCAA
	vasaseqLo3	CTCCAGCACCTCTTGATCC
Pl10	race_pl10_up1	GGTGGAGGAGCCCGACAAGA
	race_pl10_up2	AATGCTTCTGGTCCGGATTC
	race_pl10_lo1	CCAGCACGGGGGTCTTTCC
	race_pl10_lo2	GGGCCTCCTCTCGCTCCTTC
	pl10seqLo1	CGTAAACTACACATGGTCGC
Nanos	nan_spec_up1	TCTACACATCCCACGTCCTGA
	nan_spec_up2	AGAACGGACGAGTCTGCTGC
	nan_spec_lo1	TCCGTGGGCTCCGCAGTTT
	nan_spec_lo2	GACATTTGTACGCGCGCAGAA
	nanosUp1	TGYGTNTTYTGYMGNAAYAAY
	nanosUp2	TAYACNTGYCCNATHTGYGGNGC
	nanosLo1	GGRCARTAYTTDATNGTRTGNGC
	nanosLo2	GCNCCRCADATNGGRCANGTRTA
	pdnos spec fw xba1	CCTCTAGACGCTTCGATCCAAGTTACTGCAG
	pdnos spec rev hindIII	CCCAAGCTTGAAGATTCCAAACGGGGGTC
Hunchback	HbspecUp2	GCAGCAAGCCCTTCAAGTGCAA
	HbspecLo2	GGCGCATCGGTACTGGTAGAC
	HbspecUp1	CACCTGAGGAACCACTTCGGCAGC
	HbspecLo1	GCAGTCGGCGCATCGGTACTGGTA
	HbrtpcrUp	CCATGTCAAGTGATGAAGGC
	HbrtpcrLo	AACATAGCTGCAGAGATGGC

Par1	Par-1_spec_U1	ACAGCTAGAGAGGTTGCCA
	Par-1_spec_U2	GACAAGGCTCAGTTGAATCC
	Par-1_spec_L1	GGCGTGAACTCATTGCTAA
	Par-1_spec_L2	GCTAAACCCAAAGTCTGC
Actin	actin_fw2	GGAGACGATGCTCCCAGAGCT
	actin_rev2	AACTCTCAATTCATTGTAGAAGGTGT
Otx	PdOtxspecU1	ACTTGCCAGAATCCAGAGTTCAGG
	PdOtxspecU2	ACAGGCGAGCCAAGTGCAGA
	PdOtxspecL1	GGGACCATATTGAGGGCGAGTT
Gbx	gbxspecUp1	CGTGGGAGCAACTTTTGGAACTAGA
	gbxspecLo1	GATTTTGACTTGGACCTCGCTCA
	gbxspecUp2	GGAACTAGAAAAAGAATTCCACAGCA
	gbxspecLo2	TCAATTTTAGATTATGAGCTATCTGAGA
Pax258	pax258specU1	TAAATGGAAGACCTCTCCCAGACGTG
	pax258specU2	AGTTAGCCCACCAAGGAGTCCGACC
	pax258specL1	GCTTTTTCTGCTGCTTTGTTTCTCACA
	pax258specL2	CGCTTGGGACATTTTCTTGATCACACA

Special primers and parameters used for cloning and/or RT-PCR

… for *vasa* cloning

RNA was isolated from 48hpf *P. dumerilii*, and a 443bp *Pd-vas* fragment was obtained with degenerate primers designed to match conserved regions (Figure 5A): DLMACAQT (5'-gay ytn atg gcn tgy gcn car ac-3', blue arrow-a), ATFPEEIQ (5'-gcn acn tty ccn gar gar ath ca-3', blue arrow-b) and IDDYVHRIGR (5'-ckn ccd atn ckr tgn acr tan tcr tcd at-3', blue arrow-c). Specific primers (5'-gcc act ggg gag atc gta -3', red arrow-d and 5'-gca gcc act gag gta gca a-3', red arrow-e) were then used to obtain part of the 5' end of the gene. The 3' end of the gene was obtained form the *P. dumerilii* 48hpf cDNA library, located at EMBL, Heidelberg.

… for pl10 cloning

A 446bp fragment of *Pd-pl10* was cloned with the same degenerated primers as used for *Pd-vas*. RACE PCR to the 3' end was performed using the specific primers (5'- ggtggaggagcccgacaaga -3', purple arrow-f and 5'- aatgcttctggtccggattc -3', purple arrow-g), and to the 5' end (5'- ccagcacgggggtctttcc -3', purple arrow-h and 5'- gggcctcctctcgctccttc -3', purple arrow-i), the position of each primer is shown in Figure 5A.

4.12 Southern-blots

For Southern-blots were done according to the protocol of (Sambrook et al., 1989). Denaturising time was reduced to 20min. The nylon membrane utilized was positively charged (Hybond-XL, Amersham Biosciences) and the samples were cross-linked by UV.

4.13 Radioactive hybridization of DNA blots

4.13.1 Synthesis of radioactive probe

The template for the probe was a fragment of an existing gene from *Platynereis*. The plasmid containing this fragment was digested out and gel eluted with GFX™ PCR DNA and Gel Band Purification Kit (Amersham Bioscience), after that it was labelled with Megaprime DNA Labelling Kit (Amersham Bioscience, #RPN 1604) and P^{32}dCTP radioactive nucleotide.

4.13.2 Hybridization procedure

The filter blots were shortly rinsed with 1xSSC and prehybridized for 15 min at 65°C in RapidHyb Buffer (Amersham Bioscience, #RPN 1636). The buffer was replaced for a fresh one, then half of the synthesized probe was added taking care of denaturing it beforehand for 5min at 95°C. After hybridizing for 1 to 2 1/2 hrs, the blot was rinsed twice with 2xSSC containing 0.1%SDS, this was followed by a longer wash in 0.1xSSC containing 0.1% SDS of half an hour at 65°C. The blot was then exposed with an intensifier screen at -80°C until a clear signal was detected.

4.14 Sequence analysis

Sequencing reactions were performed with SP6/T7 primers and processed on an automated sequencer at Genomics Core Facility (EMBL, Heidelberg), and some at Medigenomix

(Germany). The sequences obtained were first tested with BLASTX and BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/) to check for closest likely orthologs and to rule out that the gene was cloned by mistake from other organism than *Platynereis d.* DNA sequences were aligned by using SeqMan II and checked by eye for ambiguities. Protein sequences of a selected number of species were obtained from the NCBI database and aligned using CLUSTALX (Thompson et al., 1997). These alignments were used to calculate a 1000fold bootstrapped phylogenetic tree using the neighbour-joining method, and all positions with gaps in the alignment were excluded, and corrected for multiple substitutions, with the program CLUSTALX (Thompson et al., 1997).

4.15 Additional standard molecular biology techniques

Standard techniques as DNA restriction digests, DNA ligations, bacterial mini preparations ('mini preps'), electro-transformation of *E. coli*, gel electrophoresis (agarose gel: 0.8-3.5% in TAE buffer) were carried out sticking to the protocols of (Sambrook et al., 1989). Gels were stained with EtBr in TAE ($1:10^4$ dilution) for 10-20min and used for gel extraction, southern blots, and/or photographic documentation. DNA was gel eluted from agarose gel by cutting the desired band with a razor blade under long-wave UV light (λ=366nm) and using the GFX™ Gel Band Purification Kit (Amersham Bioscience) according to the manufacturer's instructions. DNA solutions were cleaned form enzymes, primers or buffers with PCR Nucleotide Removal Kit (Qiagen); bacterial maxi preparations were done using Maxi prep Kit (Qiagen), in both cases the samples were handled according to the manufacturer's protocol.

4.16 Regeneration procedure

Worms were transferred to a 1+1 mixture of natural seawater and a 7.5% magnesium chloride solution. After 1-2 minutes in this water, worms were then paralyzed. Using 2 bended needles, with a sharp transverse movement 1/2 to 1/3 of the posterior end of the worm was removed. Worms were immediately transferred to natural or artificial seawater. The wound would heal after one or two days, then worms could be fed again if desired. Cut tails, regenerated worms (or in some cases only the regenerated tail) were fixed according WMISH protocol described above.

4.17 Embedding

Sections presented in this work were cut using a vibratome or a microtrome, depending on what was available at that moment. Embedding was preformed on worms, which went through a whole mount in situ hybridization procedure before. These worms were kept at 4°C in 87% glycerol. To equilibrate the samples before embedding, fixed worms were transferred to a small Petri dish with 1xPTW and shaken by gently rotation at RT for 2hr. Samples were then rinsed twice in 1xPTW. After this step samples were post fixed for 45 min in 4% PFA /1xPTW to improve the elasticity of the tissue and the quality of the sections. Samples were then ready for embedding.

4.17.1 for *vibratome sections*

Gelatine solution

0.5% gelatine (Sigma, #G-1890)

0.05% Chromalaun (K-Cr-sulphate, Fulka # 60151)

Millipore water

Embedding mixture

0.49g gelatine (Sigma, #G-1890)

30g albumin (albumin bovine, Sigma, #A-4503)

20g sucrose

100ml of PBS

Moviol solution

9.6g Moviol 4-88 (Hoechst)

19.35ml Glycerol

24ml Millipore water

48ml 0.2 M Tris/HCl pH 8.2

Preparation of gelatine coated slides

For preparing the gelatine solution, gelatine was diluted in Millipore water, stirred at 37-40°C on a magnetic stirrer and cooled down at RT. Chromalaun was added to the solution

and mixed. Cleaned glass microscope slides (Normansky) were dipped into this solution only once for 3-5 min to avoid uneven coating, or baked at 70°C for two hours (to use within a period of 3 months).

Preparation of Moviol solution for protection of the coated slides

Moviol was diluted in glycerol, afterwards water was added and the mixture was stirred for 2 hours. Subsequently Tris/HCl was added to it and stirred during 10 min at 50°C. At last, the solution was centrifuged for 15 min at 5000 rpm. Aliquots of the supernatant could be stored at -20°C and thawed solution at 4°C.

Preparation of embedding mixture (gelatine/albumin)

For preparing the embedding mixture, gelatine was diluted in PBS buffer, stirred and warmed up until a homogenous solution was obtained. After the solution was cooled down to RT, albumin was added, after several hours of dissolving sucrose was added. This solution could be stored at -20°C and thawed several times.

Embedding procedure

Embedding took place in a mould with an inner lumen of approx. 1.9 x 1.2 x 1.1 cm^3. Samples were removed from 1xPTW and put in the gelatine/albumin solution for few minutes. 160µl of 25% glutaraldehyde was added to 2ml gelatine/albumin sol., stirred with the pipette tip and filled up in the mould without bubbles. The sample was carefully placed onto this layer (sample should float). Another 160µl of 25% glutaraldehyde was added to 2ml gelatine/albumin sol., stirred and filled up in the mould over the first layer working fast to allow the fusion of both layers. After 1 min the mixture hardened and after 15min cut on the vibratome. Samples that were not cut immediately were stored in saran wrap at 4°C for no longer than 4 days. Embedded sample was glued to the vibratome block with "super glue". Section thickness was 80-150µm. Sections were placed on gelatine treated microscope slides and mounted with 150µl Moviol sol. per cover slip of 24x60 mm size. Pictures were taken under Normansky optics using a Zeiss Axiophot.

4.17.2 for *paraffin sections*

Worms were first dehydrated by incubations of 1 hour with increasing levels of EtOH in PTW (70, 90, 95 and 100% of EtOH), then one hour in EtOH/Xylol (1:1) and twice in Xylol to increase the permeability of the tissue for allowing the paraffin to enter. After this

step the samples were transferred into paraffin for 1hr at 60°C, paraffin was exchanged and samples were left in paraffin overnight at 60°C. Next day samples were embedded into 2cm^3 moulds, using pre-warmed needles to orient them. Blocks were left to cool down, trimmed with a blade to produce a trapezoid shape and placed in the microtome. Sections were placed on positively charged slides (SuperFrost®Plus, Roth, #H 867). De-waxing was achieved by submerging the slides twice for 10 min in Xylol, then washes of 3 min each with decreasing amounts of EtOH in PBS (100, 90, 70, 50 and 30%) proceeded and at the end a 5 min in PBS followed. Samples were mounted in 87% glycerol and pictures were taken using a Zeiss Axiovert 200 microscope.

4.18 Further techniques established and genes investigated, but not referred to in this study

I performed double staining ISH. I synthesized dsRNA and performed parental RNA interference (RNAi) and injections into the embryo at single cell stage. Additional genes are *Pdu-otx*, *Pdu-gbx*, *Pdu-hox1*, and *Pdu-pax258*: I raced some of these genes, analysed their sequence and performed WMISH on different larval stages.

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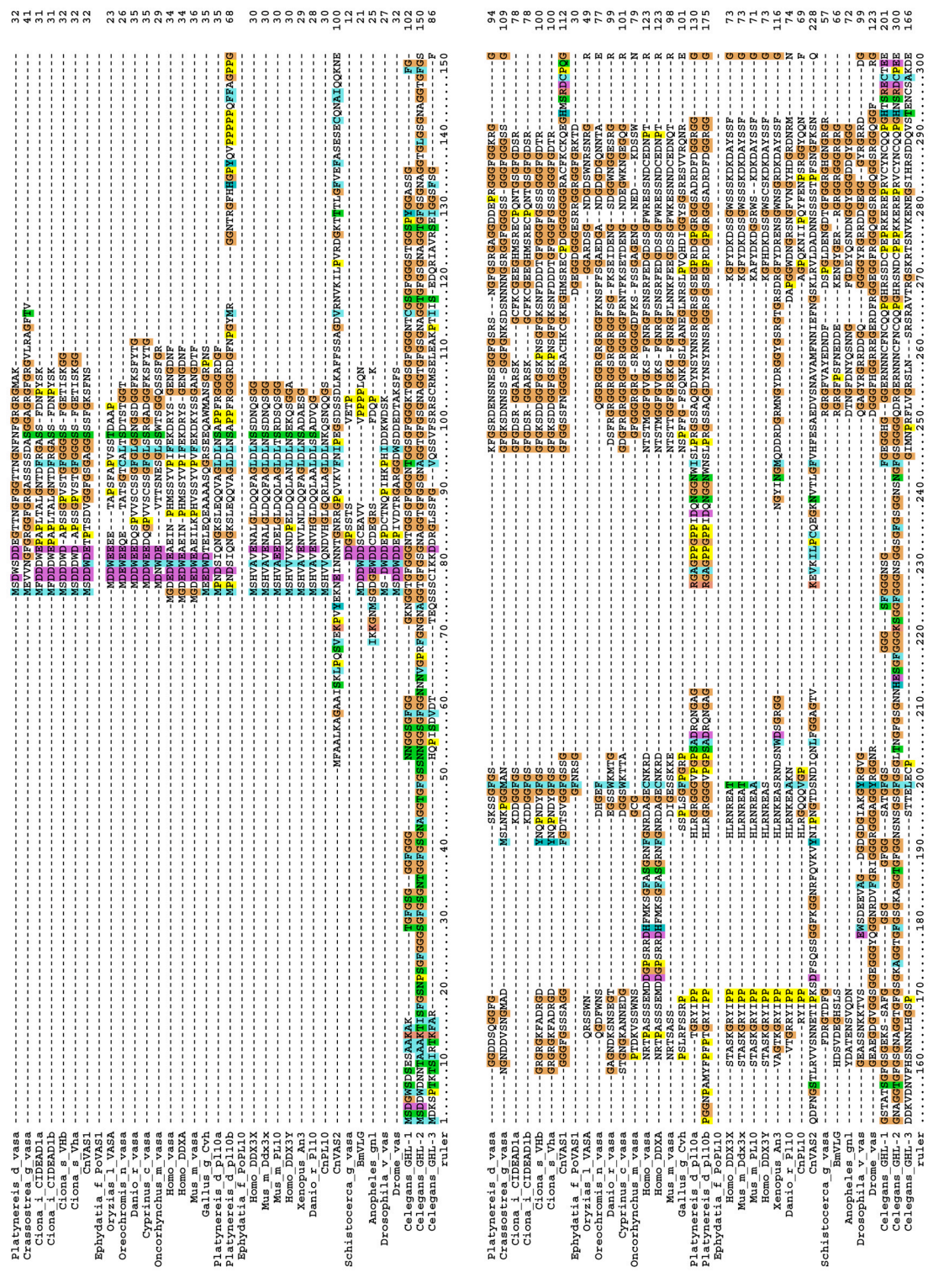
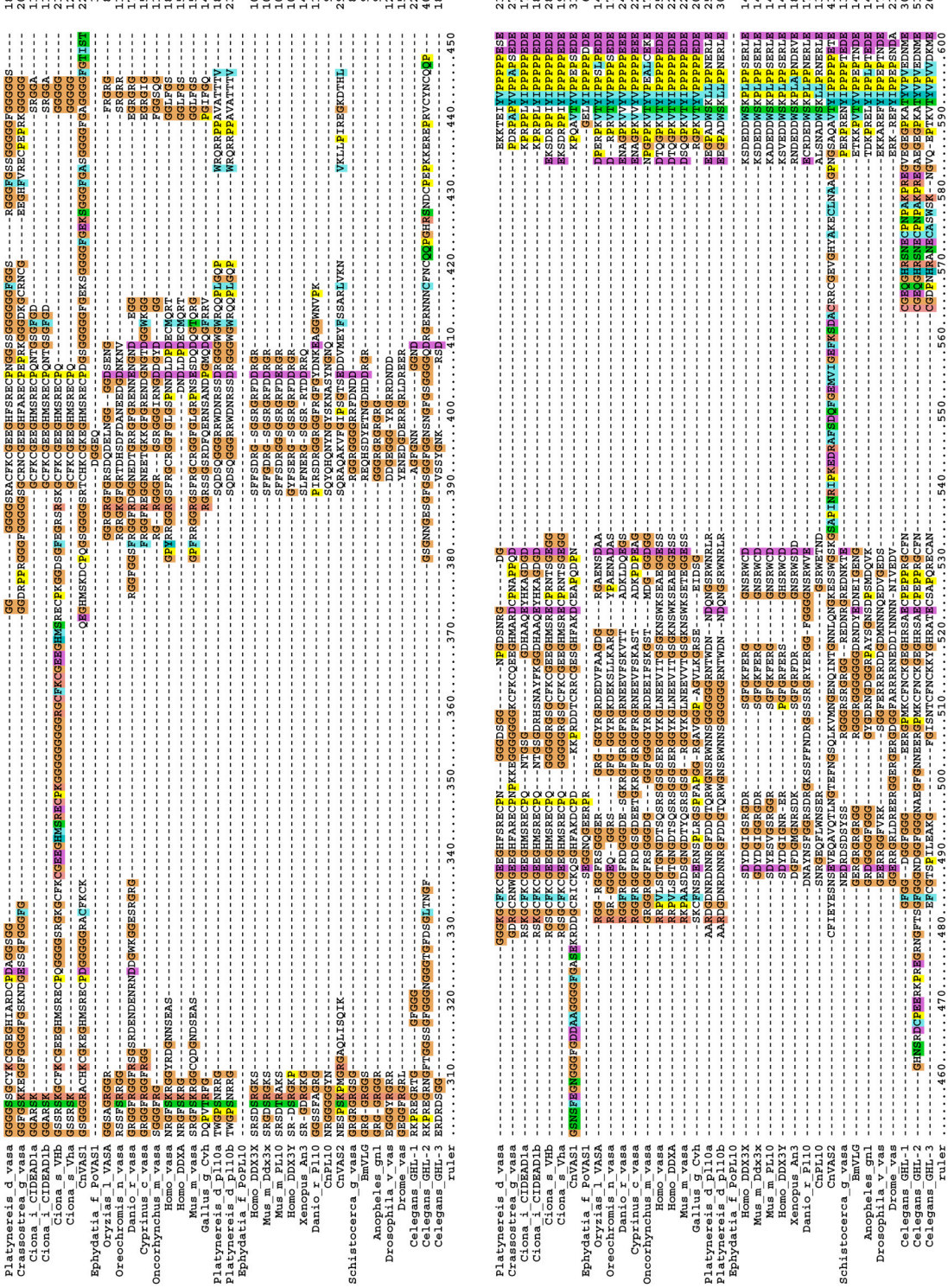
6 APPENDIX

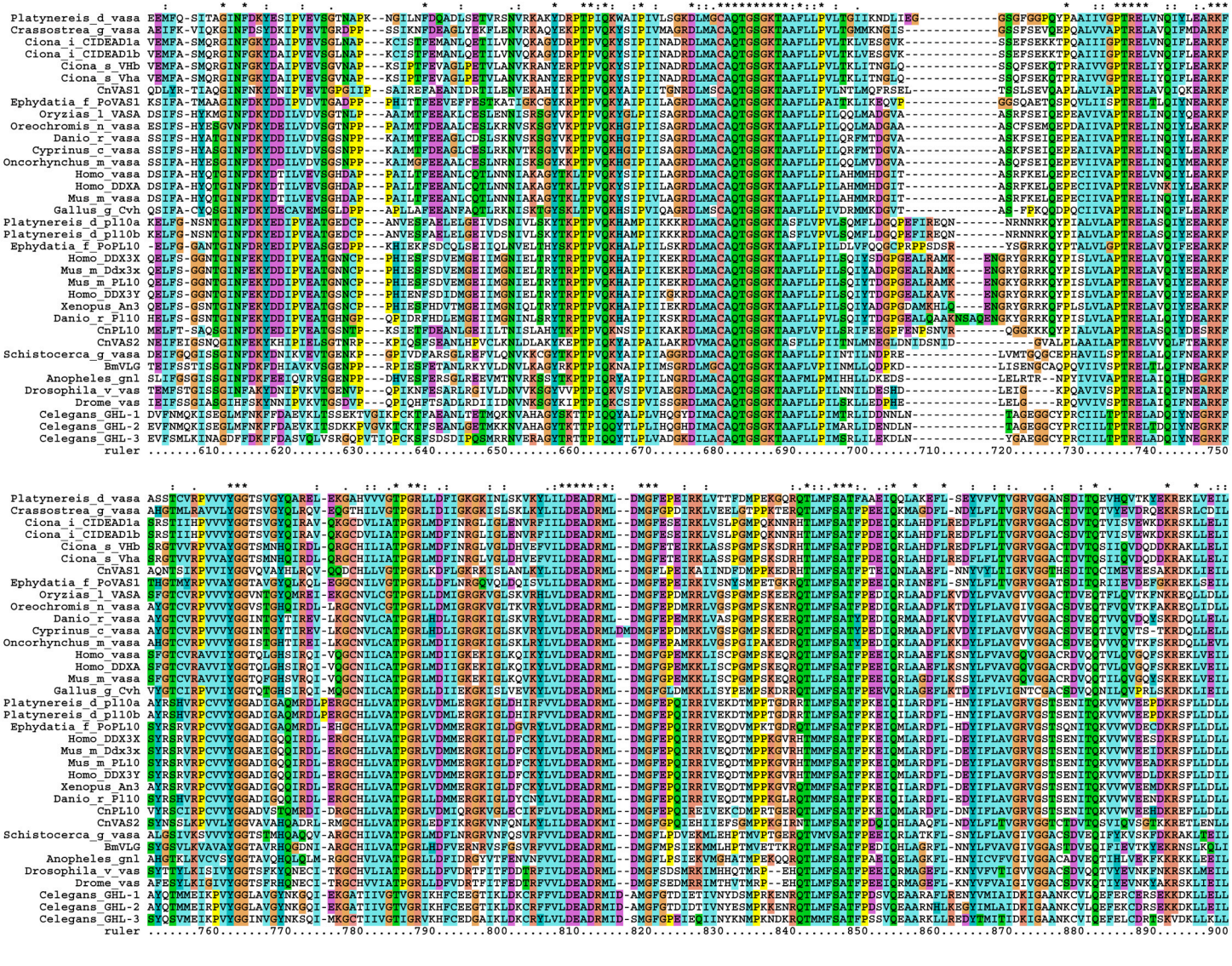
6.1 Protein sequence alignments of novel Platynereis dumerilii genes

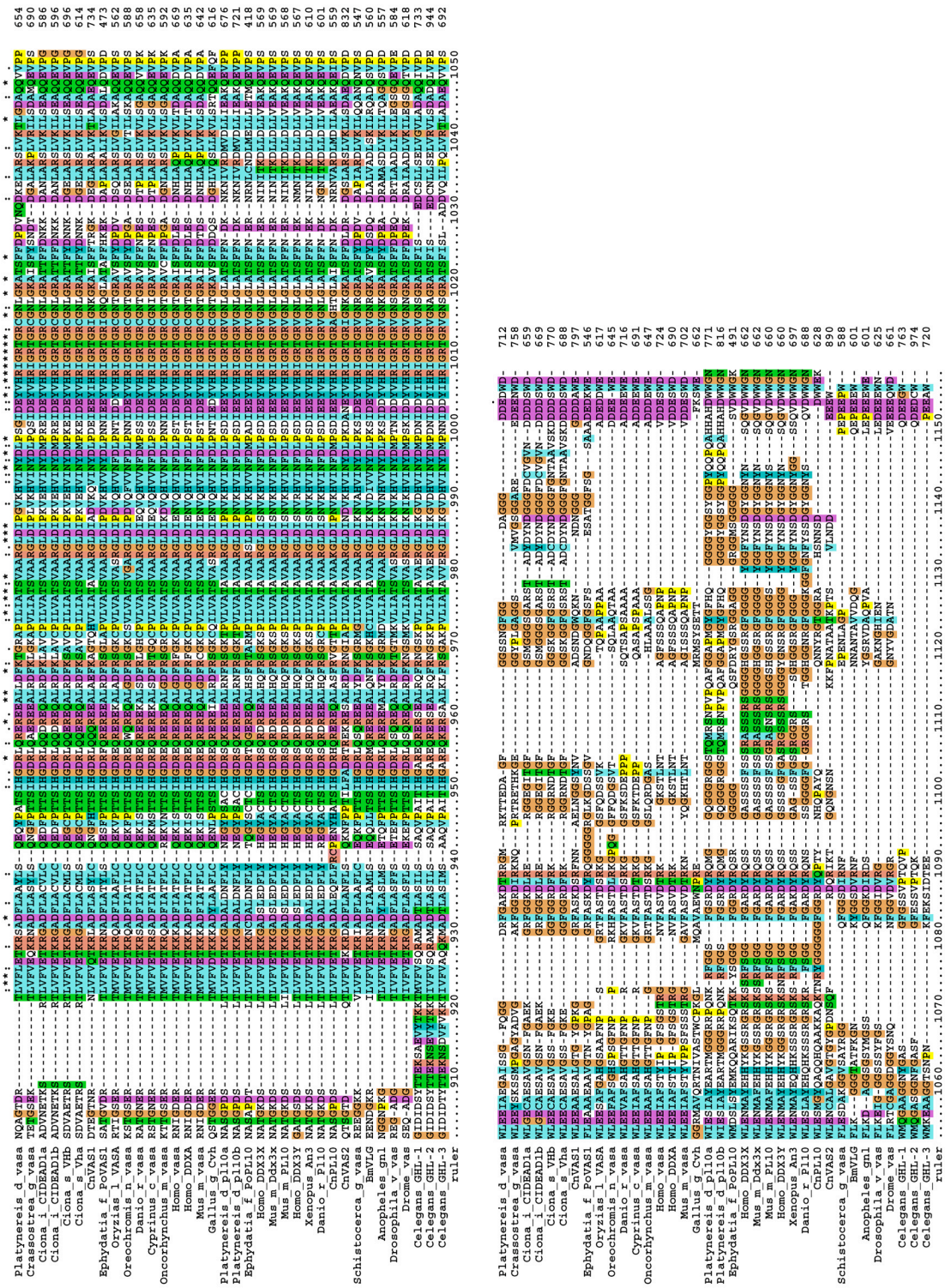
In this chapter, I included the protein sequence alignment of novel genes for *Platynereis dumerilii*. The abbreviations used for each alignment are mentioned separately in a table that contains the name of the proteins as they appear in the NCBI database (http://www.ncbi.nlm.nih.gov), and the name of the correspondent species.

6.1.1 Platynereis vasa related genes, Pdu-vas and Pdu-Pl10





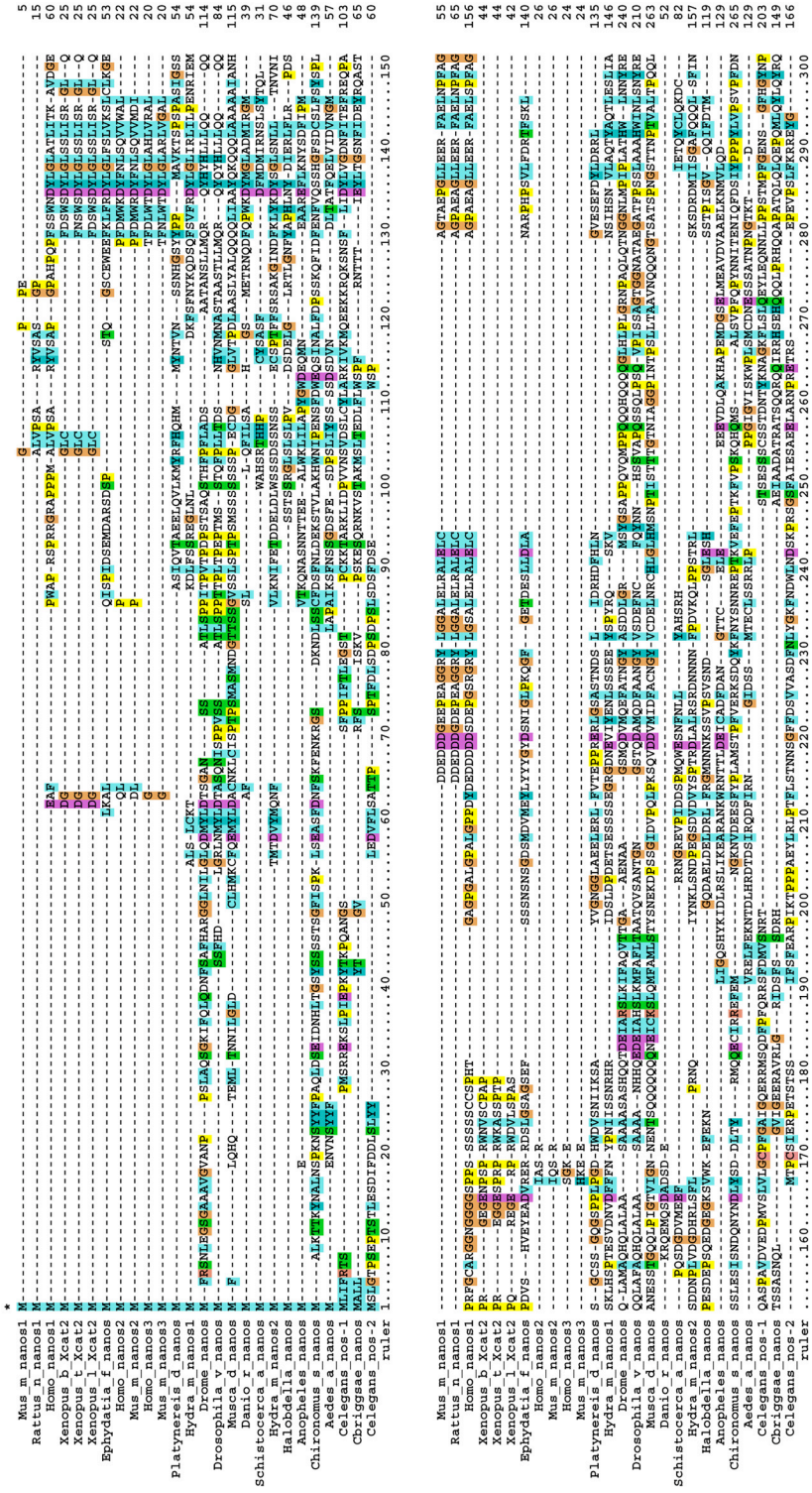


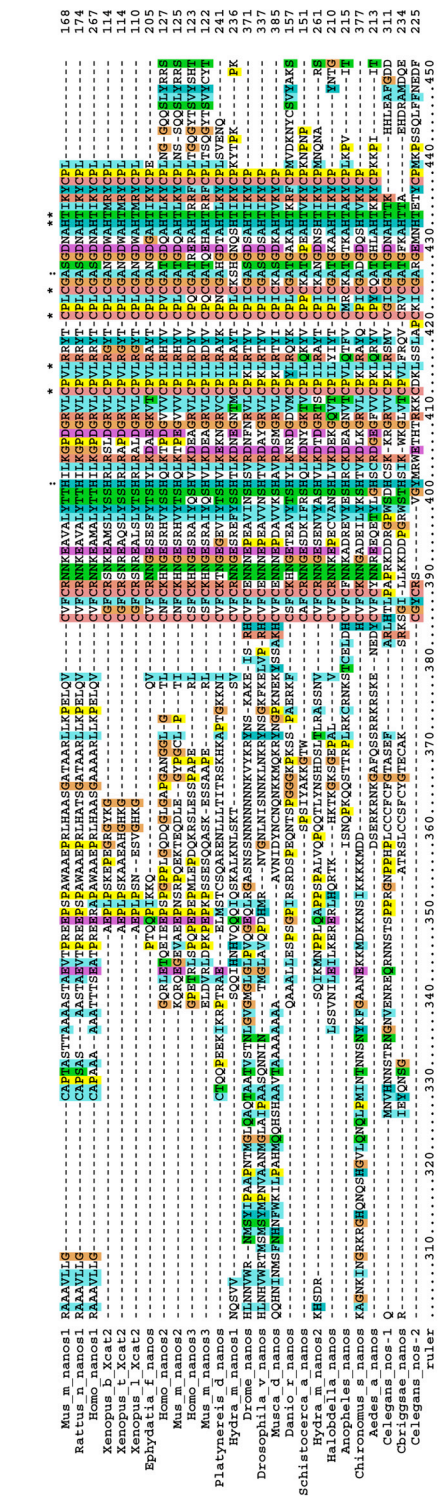


Name in AA alignment & phylogenetic tree	Species name	Name as it appears in the NCBI database
Platynereis_d_pl10a	Platynereis dumerilii	pl10 splicing variant a
Platynereis_d_pl10b	Platynereis dumerilii	pl10 splicing variant b
Ephydatia_f_PoPL10	Ephydatia fluviatilis	PL10-related protein PoPL10
Mesocricetus_a_DBX	Mesocricetus auratus	RNA helicase
Mus_m_Ddx3x	Mus musculus	embryonic RNA helicase
Homo_DDX3X	Homo sapiens	DEAD-box protein 3
Mus_m_PL10	Mus musculus	PL10 protein
Homo_DDX3Y	Homo sapiens	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3,
Xenopus_An3	Xenopus laevis	Putative ATP-dependent RNA helicase An3
Danio_r_Pl10	Danio rerio	pl10
CnPL10	Hydra magnipapillata	PL10-related protein CnPL10
Drome_bel	Drosophila melanogaster	CG9748-PA
Neurospora_c_DED1	Neurospora crassa	probable ATP-dependent RNA helicase DED1
Yeast_dep1	Schizosaccharomyces pombe	suppressor of uncontrolled mitosis
DED1p	Candida glabrata	DED1p
Yeast_Ded1p	Saccharomyces cerevisiae	ATP-dependent DEAD (Asp-Glu-Ala-Asp)-box RNA helicase; Ded1p
Yeast_DBP1	Saccharomyces cerevisiae	DBP1
DjVLGA	Dugesia japonica	DjVLGA
DjVLGB	Dugesia japonica	DjVLGB
Platynereis_d_vasa	Platynereis dumerilii	vasa
Crassostrea_g_vasa	Crassostrea gigas	vasa-like protein
CnVAS1	Hydra magnipapillata	vasa-related protein CnVAS1
Ciona_i_CIDEAD1a	Ciona intestinalis	DEAD-Box Protein

Ciona_s_VHb	Ciona savignyi	vasa homolog
Ciona_s_Vha	Ciona savignyi	vasa homolog
Ephydatia_f_PoVAS1	Ephydatia fluviatilis	Vasa-related protein PoVAS1
Oryzias_l_VASA	Oryzias latipes	VASA
Oreochromis_n_vasa	Oreochromis niloticus	vasa
Danio_r_vasa	Danio rerio	vasa homolog; vasa-like gene
Cyprinus_c_vasa	Cyprinus carpio	DEAD box RNA helicase Vasa
Oncorhynchus_m_vasa	Oncorhynchus mykiss	Vasa
Homo_vasa	Homo sapiens	VASA protein
Homo_DDXA	Homo sapiens	DDX4 protein
Mus_m_vasa	Mus musculus	mvh /m'vasa
Gallus_g_Cvh	Gallus gallus	Cvh
Xenopus_VLG1	Xenopus laevis	DEAD box protein
CnVAS2	Hydra magnipapillata	vasa-related protein CnVAS2
BmVLG	Bombyx mori	DEAD family RNA helicase
Copidosoma_f_vasa	Copidosoma floridanum	vasa protein
Schistocerca_g_vasa	Schistocerca gregaria	helicase RM62-like protein E
Drosophila_v_vas	Drosophila virilis	DEAD-box RNA helicase
Drome_vas	Drosophila melanogaster	CG3506-PA
Anopheles_gnl	Anopheles gambiae	ENSANGP00000013029
Celegans_GHL-1	Caenorhabditis elegans	Germ-Line Helicase GLH-1
Celegans_GHL-2	Caenorhabditis elegans	Germ-Line Helicase GLH-2
Celegans_GHL-3	Caenorhabditis elegans	Germ-Line Helicase GLH-3
Celegans_GHL-4	Caenorhabditis elegans	Germ-Line Helicase GLH-4

6.1.2 Platynereis Nanos

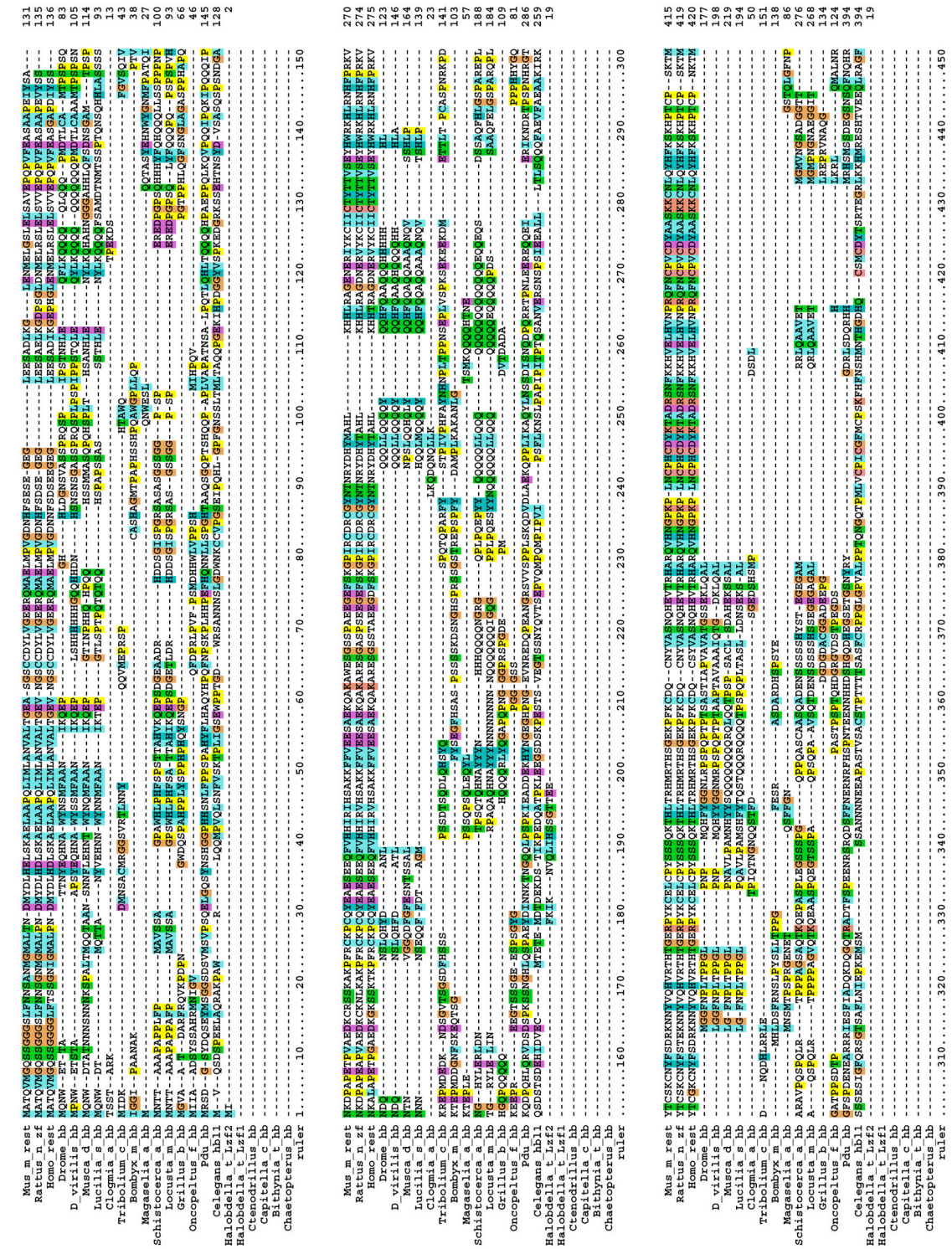


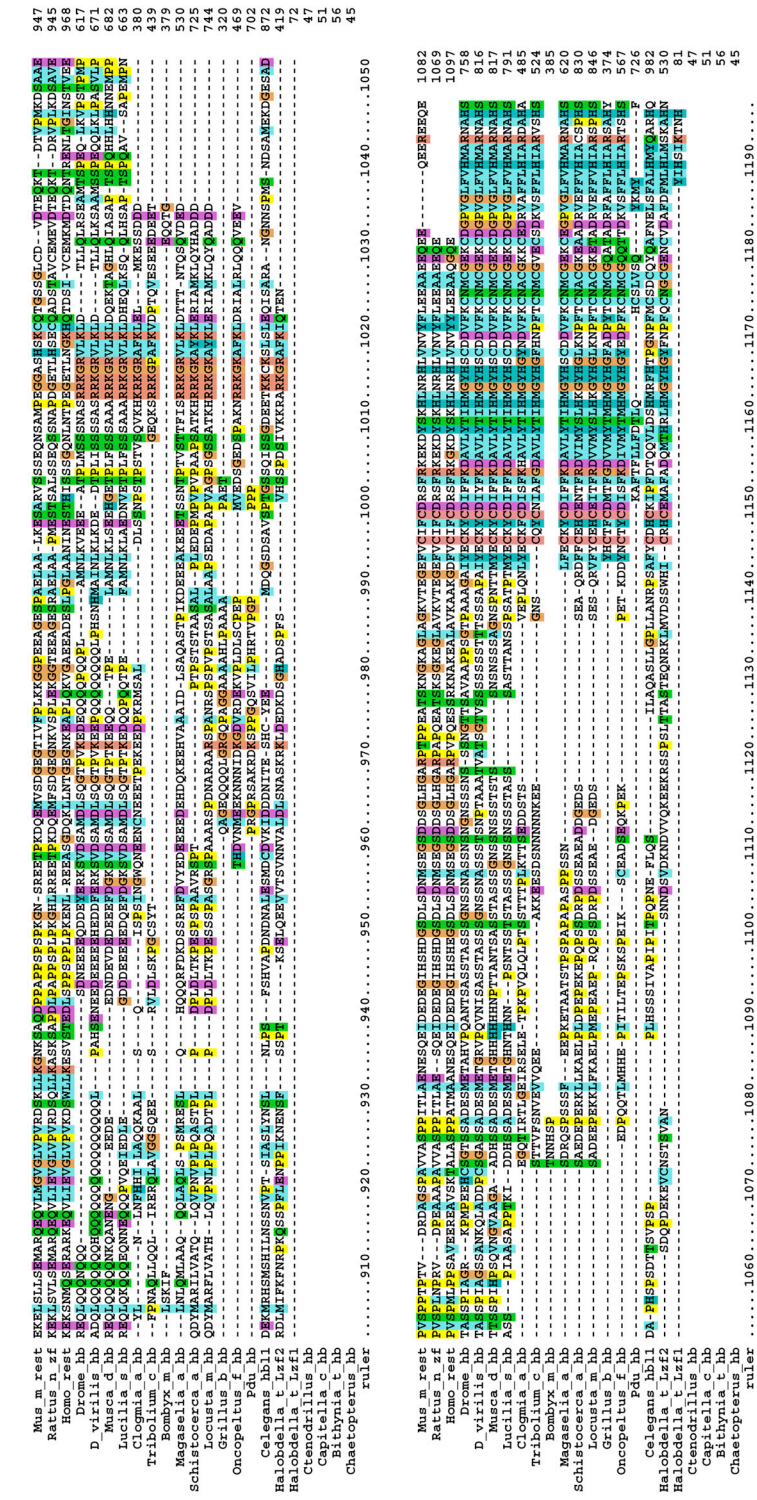


Name in AA alignment & phylogenetic tree	Species name	Name as it appears in the NCBI database
Mus_m_nanos1	Mus musculus	Nanos homolog 1
Rattus_n_nanos1	Rattus norvegicus	similar to Nanos1
Homo_nanos1	Homo sapiens	nanos homolog 1
Xenopus_b_Xcat2	Xenopus borealis	Xcat-2
Xenopus_t_Xcat2	Xenopus tropicalis	Xcat-2 protein
Xenopus_l_Xcat2	Xenopus laevis	gene Xcat-2 protein
Ephydatia_f_nanos	Ephydatia fluviatilis	Nanos-related protein PoNOS
Homo_nanos2	Homo sapiens	Nanos homolog 2
Mus_m_nanos2	Mus musculus	Nanos homolog 2
Homo_nanos3	Homo sapiens	Nanos homolog 3
Mus_m_nanos3	Mus musculus	Nanos homolog 3
Platynereis_d_nanos	Platynereis dumerilii	Nanos homolog
Hydra_m_nanos1	Hydra magnipapillata	CNNOS1
Drome_nanos	Drosophila melanogaster	CG5637-PA
Drosophila_v_nanos	Drosophila virilis	nanos
Musca_d_nanos	Musca domestica	nanos homolog
Danio_r_nanos	Danio rerio	nanos homolog
Schistocerca_a_nanos	Schistocerca americana	nanos
Hydra_m_nanos2	Hydra magnipapillata	CNNOS2
Helobdella_nanos	Helobdella robusta	Hro-nos
Anopheles_nanos	Anopheles gambiae	NOS protein
Chironomus_s_nanos	Chironomus samoensis	nanos homolog
Aedes_a_nanos	Aedes aegypti	NOS protein
Celegans_nos-1	Caenorhabditis elegans	NanOS related (nos-1)
Cbriggsae_nanos	Caenorhabditis briggsae	Hypothetical protein CBG20913

Celegans_nos-2	Caenorhabditis elegans	NanOS related (nos-2)

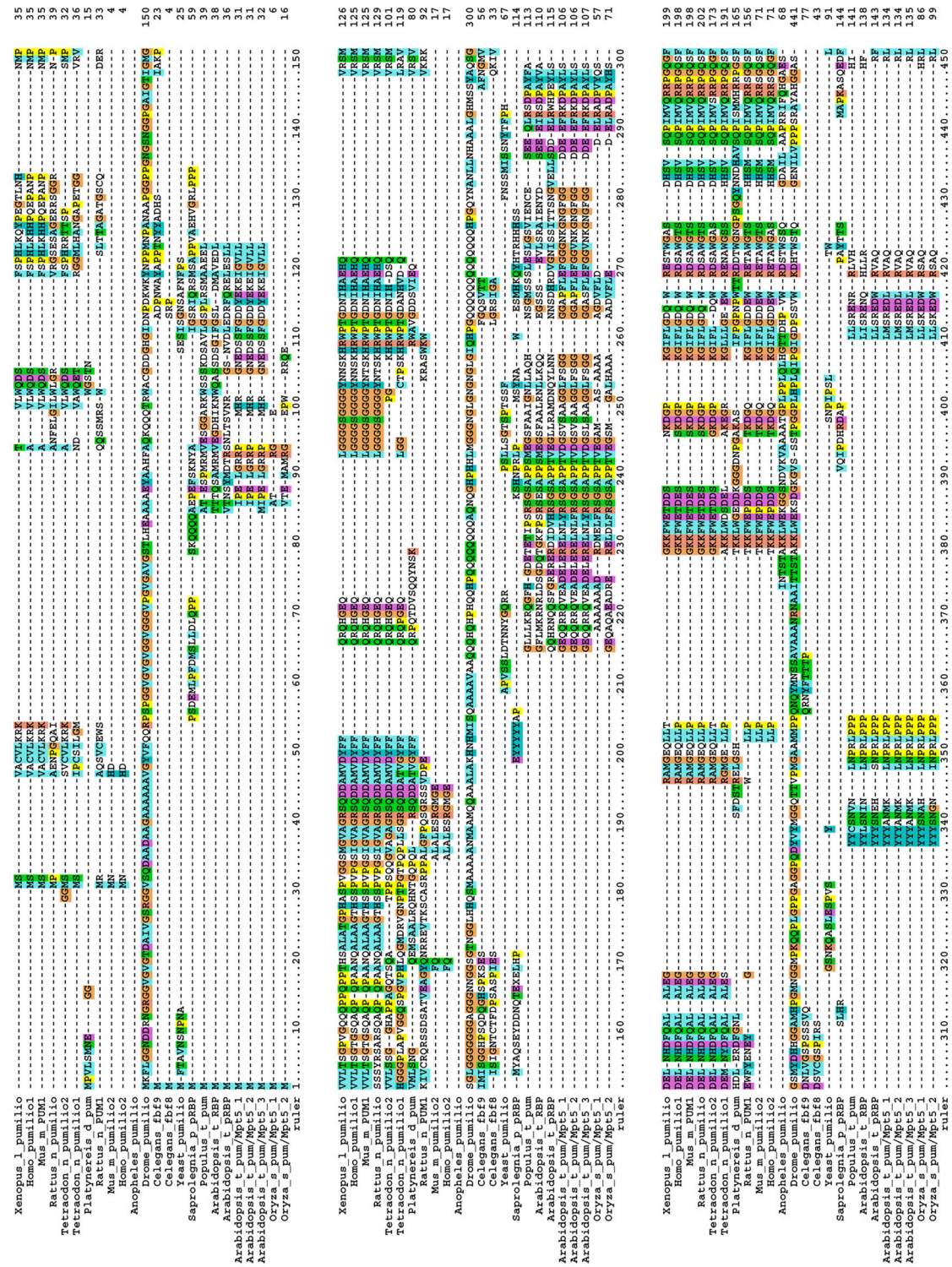
6.1.3 Platynereis Hunchback

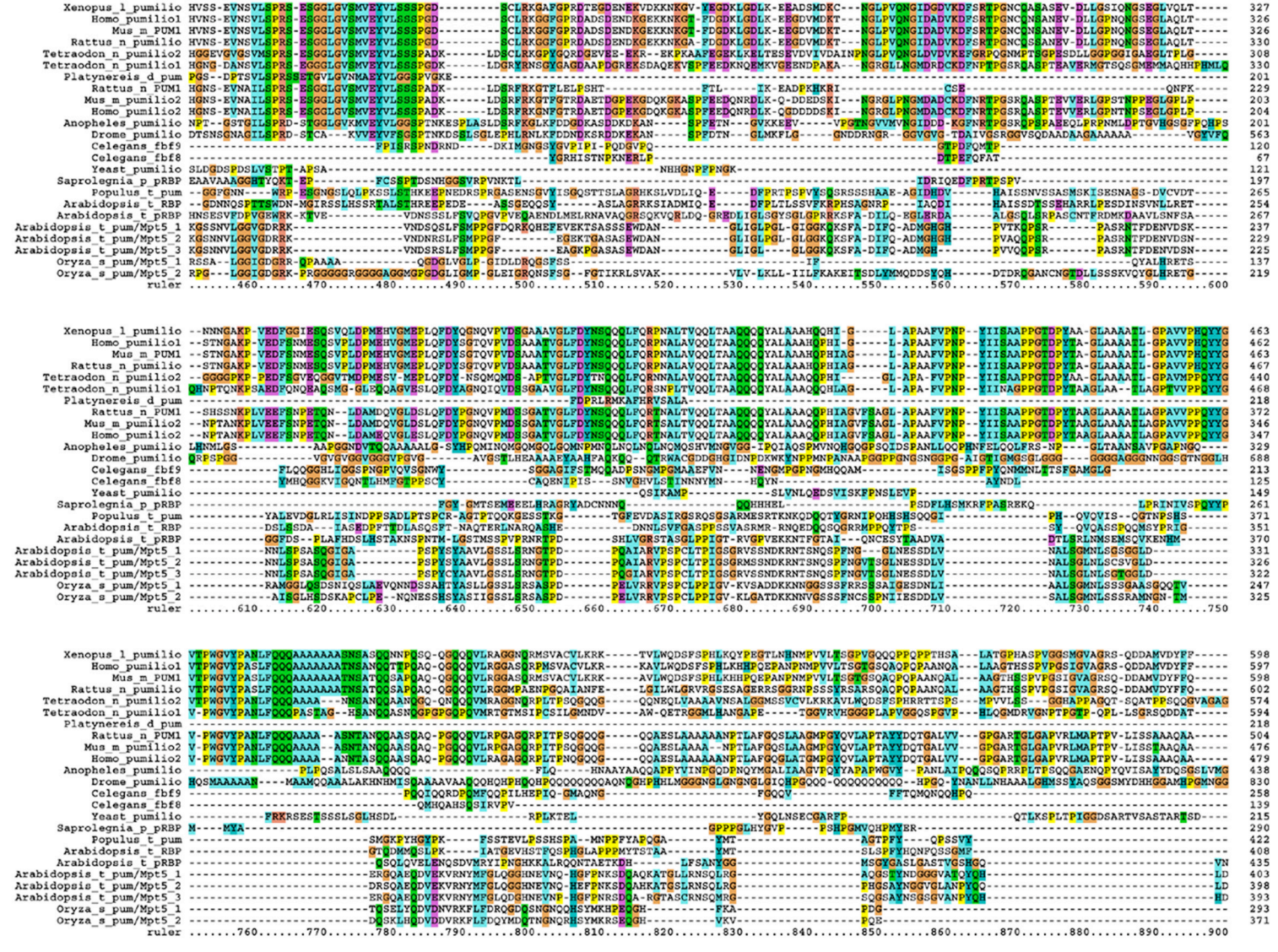


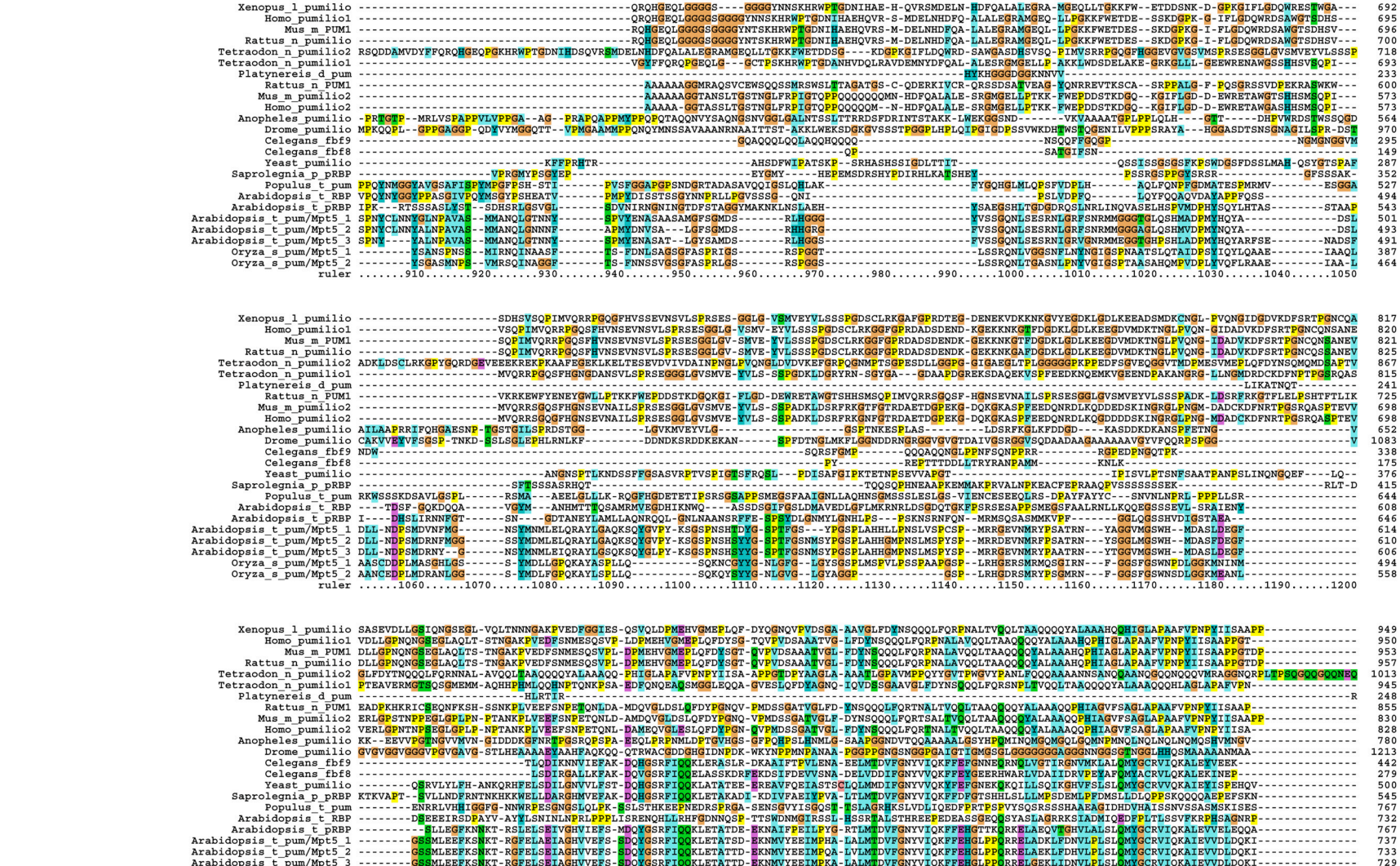


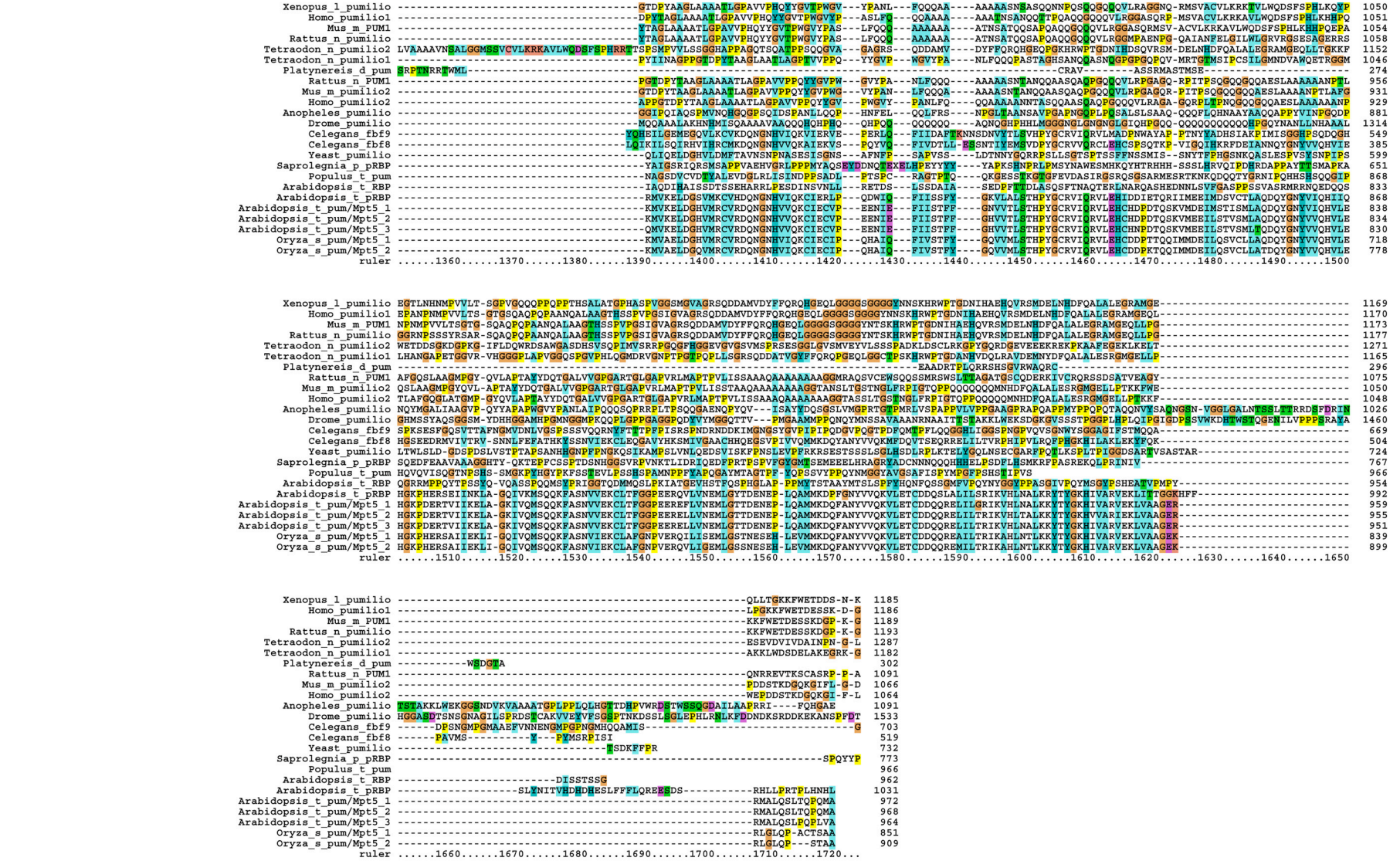
Name in AA alignment & phylogenetic tree	Species name	Name as it appears in the NCBI database
Mus_m_rest	Mus musculus	RE1-silencing transcription factor
Rattus_n_zf	Rattus norvegicus	zinc finger transcription factor REST protein
Homo_rest	Homo sapiens	RE1-silencing transcription factor
Drome_hb	Drosophila melanogaster	finger protein hunchback - fruit fly
D_virilis_hb	Drosophila virilis	gap protein hunchback - fruit fly
Musca_d_hb	Musca domestica	hunchback zinc finger protein
Lucilia_s_hb	Lucilia sericata	Hunchback protein
Clogmia_a_hb	Clogmia albipunctata	Hunchback protein
Tribolium_c_hb	Tribolium castaneum	hunchback
Bombyx_m_hb	Bombyx mori	Hunchback protein
Magaselia_a_hb	Megaselia abdita	putative Hunchback protein
Schistocerca_a_hb	Schistocerca americana	transcription factor hunchback
Locusta_m_hb	Locusta migratoria	Hunchback protein
Grillus_b_hb	Gryllus bimaculatus	hunchback
Oncopeltus_f_hb	Oncopeltus fasciatus	hunchback
Pdu_hb	Platynereis dumerilii	Hunchback protein
Celegans_hbl1	Caenorhabditis elegans	hunchback-related protein
Helobdella_t_Lzf2	Helobdella triserialis	leech zinc finger protein
Helobdella_t_Lzf1	Helobdella triserialis	leech zinc finger protein
Ctenodrillus_hb	Ctenodrillus serratus	hunchback
Capitella_c_hb	Capitella capitata	hunchback
Bithynia_t_hb	Bithynia tentaculata	Hunchback protein
Chaetopterus_hb	Chaetopterus sp. RMS-2000	hunchback

6.1.4 Platynereis Pumilio



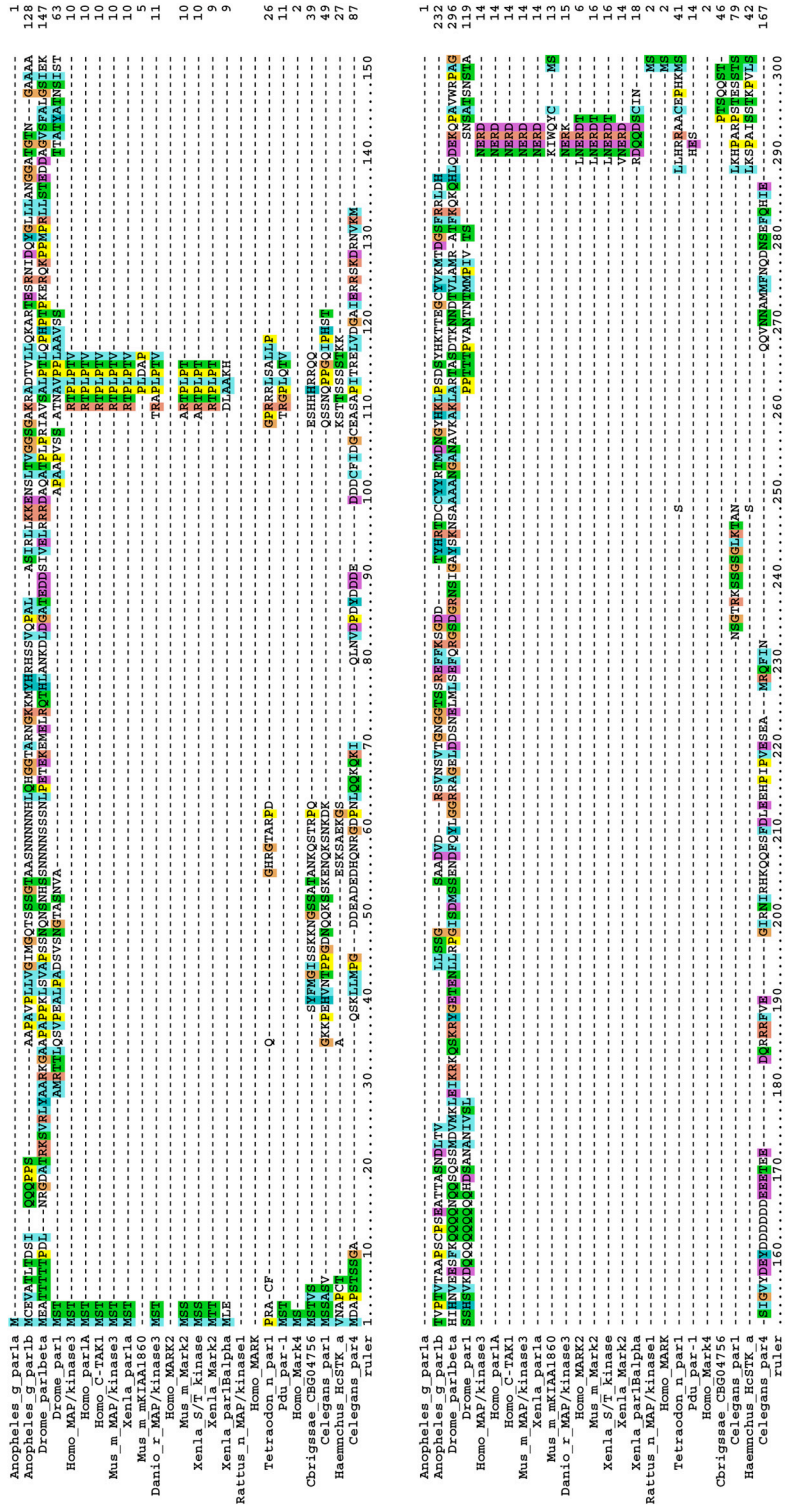


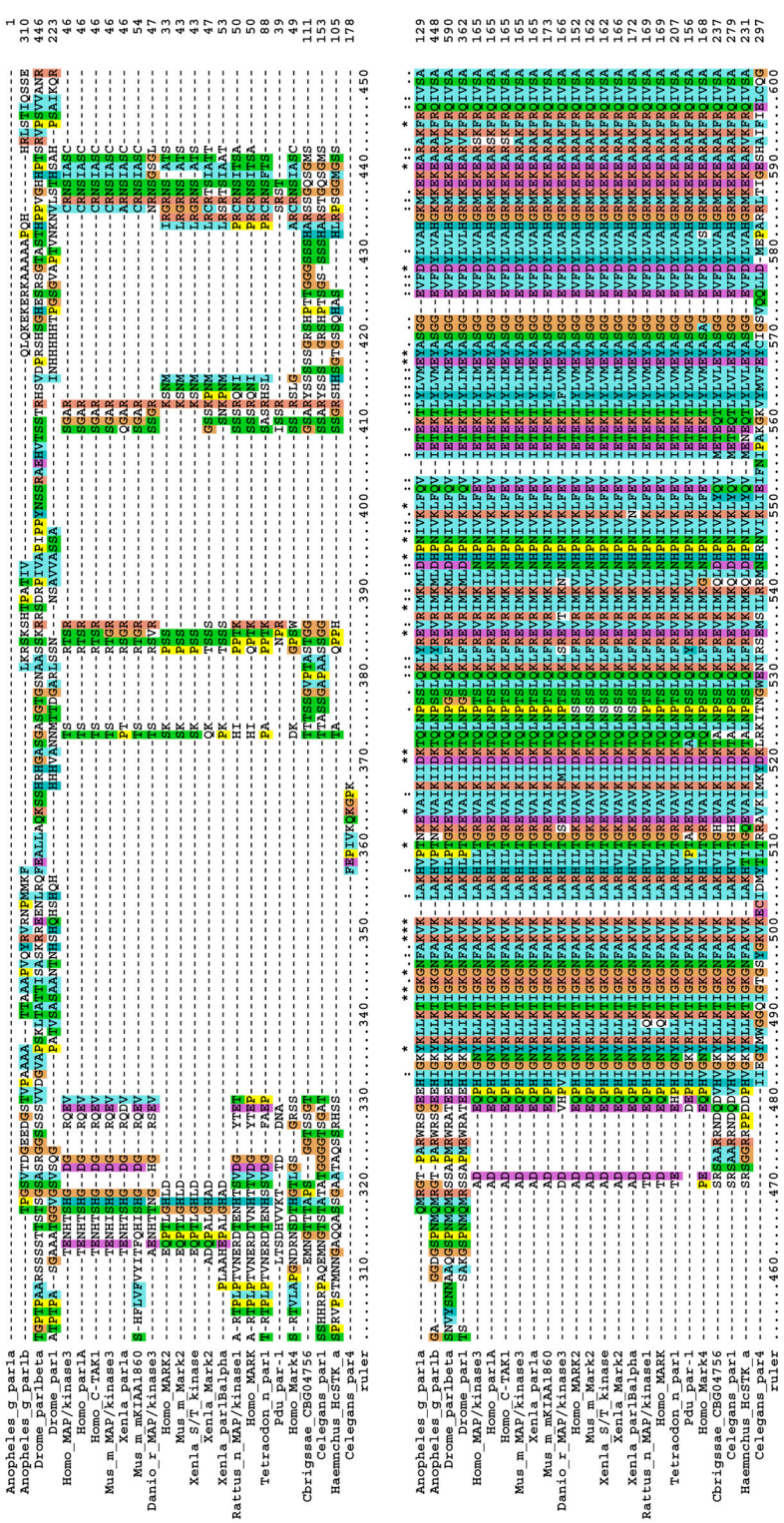


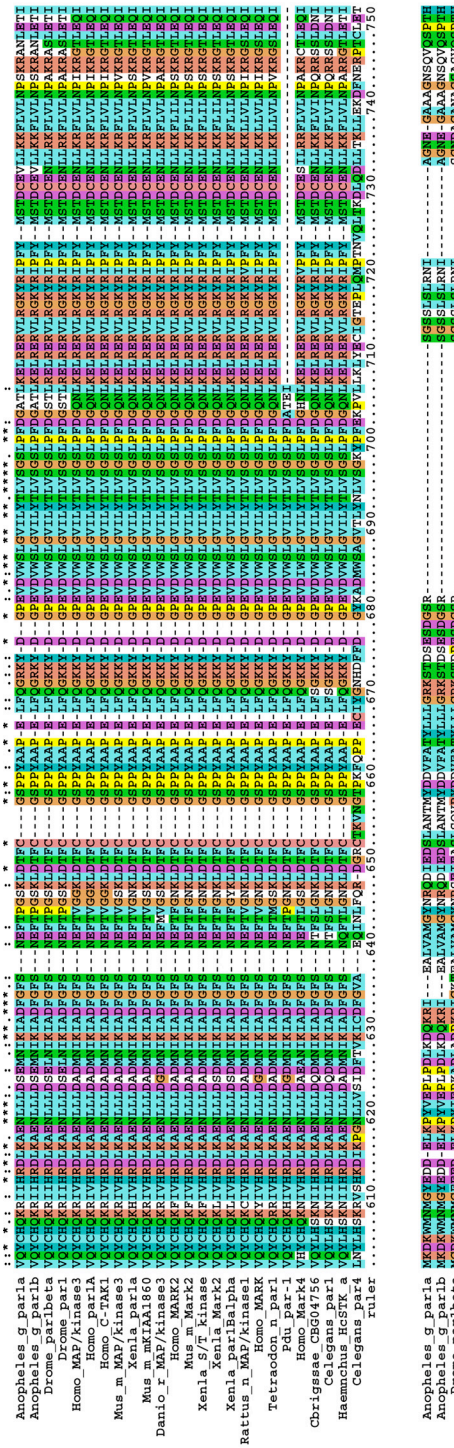


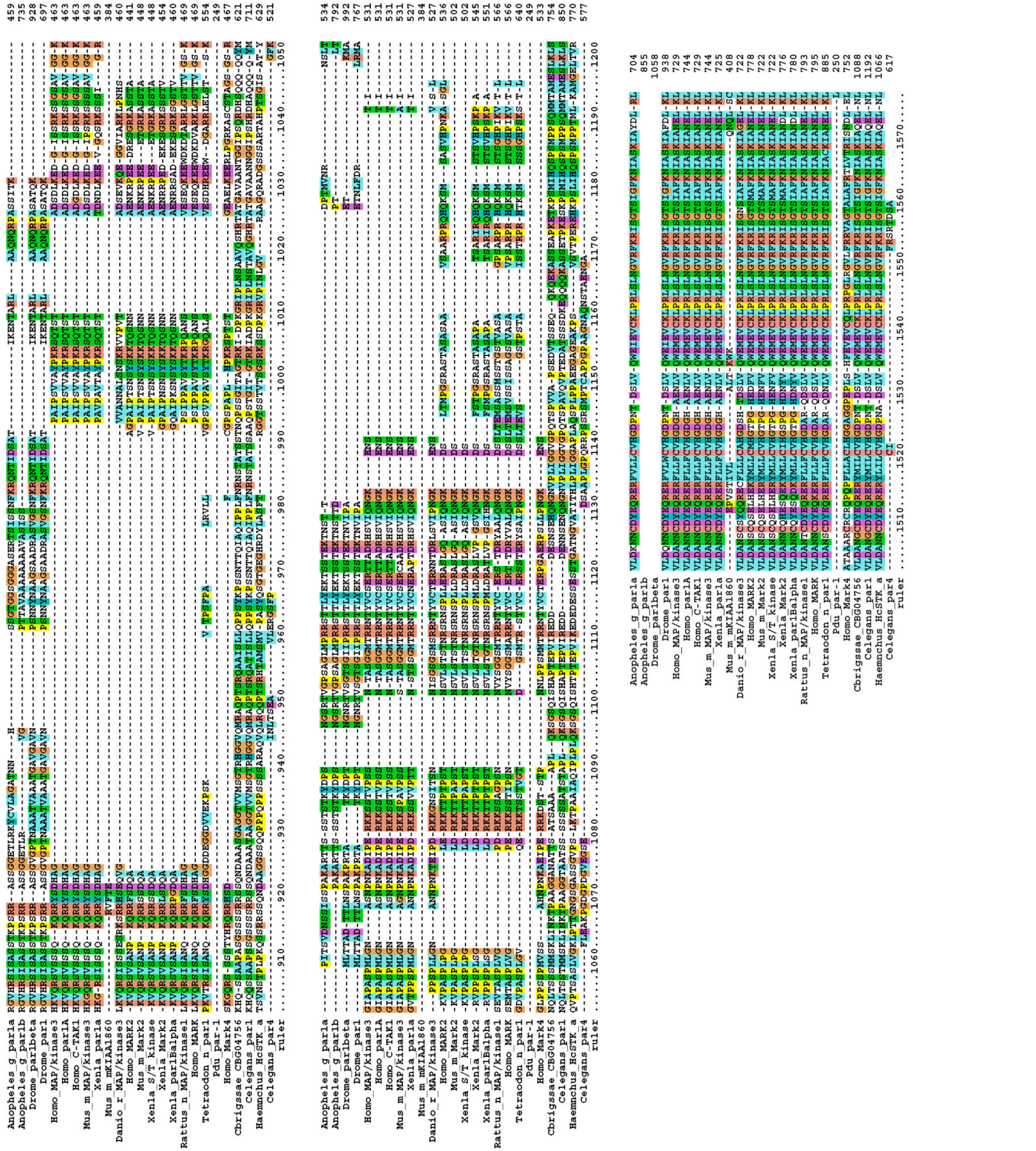
Name in AA alignment & phylogenetic tree	Species name	Name as it appears in the NCBI database
Xenopus_l_pumilio	Xenopus laevis	Pumilio
Homo_pumilio1	Homo sapiens	Pumilio homolog 1
Mus_m_PUM1	Mus musculus	Pumilio homolog 1
Rattus_n_pumilio	Rattus norvegicus	similar to mKIAA0099 protein
Tetraodon_n_pumilio2	Tetraodon nigroviridis	unnamed protein product
Tetraodon_n_pumilio1	Tetraodon nigroviridis	unnamed protein product
Platynereis_d_pum	Platynereis dumerilii	Pumilio homolog
Rattus_n_PUM1	Rattus norvegicus	similar to PUM1
Mus_m_pumilio2	Mus musculus	Pumilio homolog 2
Homo_pumilio2	Homo sapiens	Pumilio 2
Anopheles_pumilio	Anopheles gambiae	ENSANGP00000002715
Drome_pumilio	Drosophila melanogaster	abdominal segment formation protein pumilio
Celegans_fbf9	Caenorhabditis elegans	Puf (pumilio/fbf) domain-containing protein 9
Celegans_fbf8	Caenorhabditis elegans	Puf (pumilio/fbf) domain-containing protein 8
Yeast_pumilio	Schizosaccharomyces pombe	pumilio family protein [imported] - fission yeast
Saprolegnia_p_pRBP	Saprolegnia parasitica	putative RNA binding protein
Populus_t_pum	Populus tremula	pumilio domain-containing protein PPD1
Arabidopsis_t_RBP	Arabidopsis thaliana	RNA binding protein-like
Arabidopsis_t_pRBP	Arabidopsis thaliana	putative RNA binding protein
Arabidopsis_t_pum/Mpt5_1	Arabidopsis thaliana	putative pumilio/Mpt5 family RNA-binding protein
Arabidopsis_t_pum/Mpt5_2	Arabidopsis thaliana	putative pumilio/Mpt5 family RNA-binding protein
Arabidopsis_t_pum/Mpt5_3	Arabidopsis thaliana	putative pumilio/Mpt5 family RNA-binding protein
Oryza_s_pum/Mpt5_1	Oryza sativa	putative pumilio/Mpt5 family RNA-binding protein
Oryza_s_pum/Mpt5_2	Oryza sativa	P0518C01.14

6.1.5 Platynereis Par-1





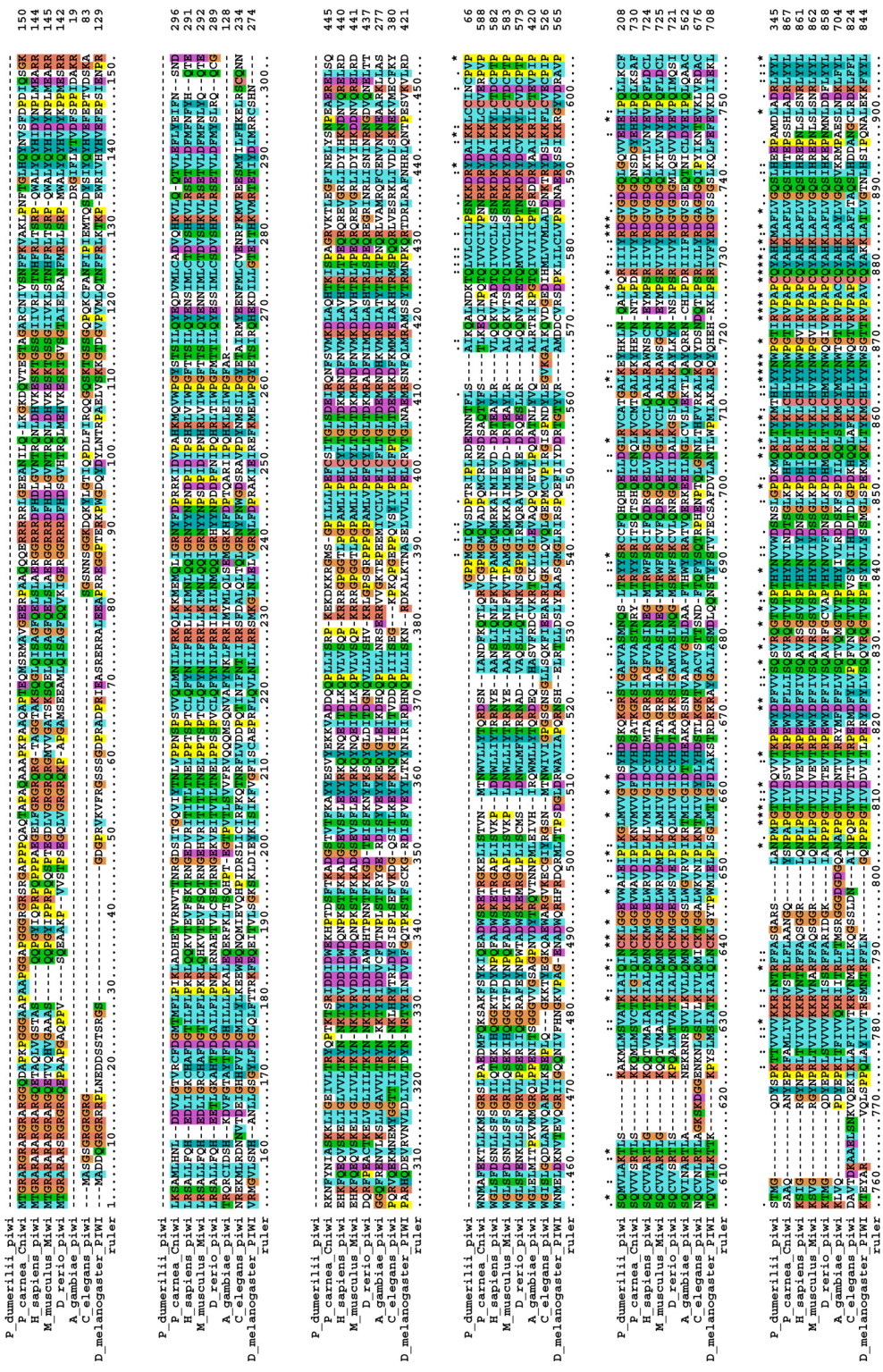




Name in AA alignment & phylogenetic tree	Species name	Name as it appears in the NCBI database
Anopheles_g_par1a	Anopheles gambiae	ENSANGP00000022382
Anopheles_g_par1b	Anopheles gambiae	ENSANGP00000016067
Drome_par1beta	Drosophila melanogaster	Ser/Thr protein kinase PAR-1beta
Drome_par1	Drosophila melanogaster	PAR-1
Homo_MAP/kinase3	Homo sapiens	MAP/microtubule affinity-regulating kinase 3
Homo_par1A	Homo sapiens	Ser/Thr protein kinase PAR-1A
Homo_C-TAK1	Homo sapiens	Cdc25C associated protein kinase C-TAK1
Mus_m_MAP/kinase3	Mus musculus	MAP/microtubule affinity-regulating kinase 3
Xenla_par1a	Xenopus laevis	Ser/Thr protein kinase PAR-1A
Mus_m_mKIAA1860	Mus musculus	mKIAA1860 protein
Danio_r_MAP/kinase3	Danio rerio	MAP/microtubule affinity-regulating kinase 3
Homo_MARK2	Homo sapiens	MARK2
Mus_m_Mark2	Mus musculus	Mark2 protein
Xenla_S/T_kinase	Xenopus laevis	serine/threonine kinase
Xela_Mark2	Xenopus laevis	Mark2
Xenla_par1Balpha	Xenopus laevis	Ser/Thr protein kinase PAR-1B alpha
Rattus_n_MAP/kinase1	Rattus norvegicus	Ser/Thr protein kinase PAR-1
Homo_MARK	Homo sapiens	MARK
Tetraodon_n_par1	Tetraodon nigroviridis	unnamed protein product
Pdu_par-1	Platynereis dumerilii	par-1
Homo_Mark4	Homo sapiens	MARK4 serine/threonine protein kinase
Cbrigssae_CBG04756	Caenorhabditis briggsae	Hypothetical protein CBG04756
Celegans_par1	Caenorhabditis elegans	par-1

HcSTK_a	Haemonchus contortus	HcSTK
Celegans_par4	Caenorhabditis elegans	par-4

6.1.6 Platynereis Piwi



Name in AA alignment & phylogenetic tree	Species name	Name as it appears in the NCBI database
P_dumerilii_piwi	Platynereis dumerilii	piwi
P_carnea_Cniwi	Podocoryne_carnea	Cniwi
H_sapiens_piwi	Homo sapiens	piwi-like1
M_musculus_Miwi	Mus musculus	Miwi protein
D_rerio_piwi	Danio rerio	piwi-like1
D_melanogaster_PIWI	Drosophila melanogaster	PIWI
C_elegans_piwi	Caenorhabditis_elegans	piwi
A_gambiae_piwi	Anopheles gambiae	ENSANGP00000006401

6.2 Definitions

Following, I provide some definitions that might be helpful to understand the context of this thesis:

archetypical	Representing or constituting an original type after which other similar things are patterned
blastema	In regeneration, the blastema is formed forms beneath the wound epidermis, and gives rise to the regenerated tissue.
blastopore	The mouth opening of the gastrula.
coelom	Inner cavity lined by mesoderm, which often forms a peritoneum.
epiboly	The process during gastrulation in which the ectoderm extends to cover the whole of the embryo.
gastrulation	The processes of cell and tissue movements whereby the cells of the blastula are rearranged to form a three-layered body plan, which consists of the outer ectoderm, inner endoderm and interstitial mesoderm.
hyposphere	The lower part of the trochophora, i.e. below the prototroch.
peritoneum	Mesodermal epithelium surrounding a coelomic cavity; also called mesothelium.
pluripotency	The potential of a cell to develop into more than one type of mature cell, depending on environment.
primordial germ cells	Germline cells at all stages of development from the time when this lineage is formed until they arrive at the gonad and start differentiating into gametes.
prototroch	Horseshoe or ring of compound (with few exceptions) cilia in front of the mouth of protostome larvae (and adult rotifers), functioning as a locomotory organ and often as downstream-collecting band in filter feeding.
triploblastic	Of, pertaining to, or designating, that condition of the ovum in which there are three primary germinal layers, or in which the blastoderm splits into three layers.

totipotency	The ability of a cell or nucleus to develop into any type of specialised cell or nucleus, i.e., the ability of a single cell to develop and differentiate into a complete organism.
Urbilateria	The common ancestors of deuterostomes and protostomes.

6.3 Abbreviations

Standard units and symbols of standard multiples are not displayed below. This is a summary of the most common abbreviations used in this thesis. I had previously introduced most of them in the text at the site of their first appearance.

a	anterior or apical
AA	amino acid(s)
A/P	Antero-posterior
apo	apical organ
ATP	Adenosine-5´triphosphate
bp	base pair(s)
BSA	bovine serum albumine
bv	blood vessel
°C	degree Celsius
C-terminus	Carboxy-terminus (of a peptide)
CTP	Cytosine-5´triphosphate
ch. s	chaetal sacs
GC(s)	germ cell(s)
GZ	growth zone
d	dorsal
Da	Dalton
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxy-A/C/G/T-trisphosphate
dpc	day post cut
dpf	days post fertilization
DTT	dithiothreitol
EST	expressed sequence tag
fb	frontal body
Fgf (FgfR)	Fibroblast growth factor (receptor)
g	gut or gravital force
GTP	Guanosine-5´-triphosphate
H	head

HMM	Hidden Markov Models
hpf	hours post fertilization
ld	lipid droplet
m	muscle
M	mol/l
mb	mesodermal bands
ml	midline
mpf	months post fertilization
mRNA	messenger RNA
nc	nerve cord
NRE	nanos response element
N-terminus	Amino-terminus (of a peptide)
ORF	Open reading frame
P	parapodia
pa	pygidial area
PGC(s)	primordial germ cell(s)
Pdu	*Platynereis dumerilii*
PCR	polymerase chain reaction
post (or p)	posterior
rpm	rotations per minute
rRNA	ribosomal RNA
RT	room temperature
s	stomodaeum
SDS	Sodium-dodekylsulfate
spc	stomodaeum precursor cell(s)
TTP	Thymidine-5´triphosphate
UTP	Uridine-5´triphosphate
UTR	Untranslated region
UV	ultraviolet
v	ventral
veg	vegetal
vp	ventral plate
WMISH	whole-mount *in situ* hybridisation
zf	zinc finger

Short Summary

Despite specific knowledge from selected model systems, a unified theory about the evolutionary origin of germ cell specification still requires detailed analyses in other species. In this thesis, I present evidence on the establishment of the germline and its correlation with pluripotency and embryonic patterning in the polychaete *Platynereis dumerilii*, an emerging model system that is well suitable for comparative developmental studies across Bilateria.

In the first section, I describe the germline development of *Platynereis* with the help of several molecular germline markers, including *vasa*, *nanos* and *par-1*. This analysis revealed that the primordial germ cells originate from the mesodermal lineage and emerge during the final stages of larval development from a region of undifferentiated cells, namely the posterior growth zone. In juveniles, the primordial germ cells remain quiescent in the former larval segments, while the polychaete grows by addition of new segments from the posterior growth zone. At the onset of gametogenesis, these cells proliferate, migrate to the posterior segments and differentiate into gametes.

I extended this analysis in the second section by investigating the expression of germline genes in two not terminally differentiated tissues: the growth zone and tail regenerates. From the expression pattern of vasa in these tissues and its comparison to several genes that are expressed in totipotent and stem cells, such as *pl10* and *piwi*, I suggest that there is an ancestral link between these processes, which is conserved in *Platynereis*.

The third part of my thesis addresses the relationship between germline determination and axis formation. Here, I compared the expression pattern of *nanos* – one of the posterior genes in *Drosophila* responsible for the maintenance of germ cell fate and establishment of the posterior segments – to the mRNA distribution of the gap gene *hunchback* in the early larva. My analysis is consistent with a conserved role of Nanos (and possibly its partner Pumilio) in the translational repression of *hunchback*, as it is the case in *Drosophila*, arguing that this system might be a conserved mechanism to establish embryonic polarity in the protostomes.

Zusammenfassung

Eine vereinheitlichende Theorie über den evolutiven Ursprung der Keimzellspezifizierung erfordert, über spezifische Studien an wenigen ausgesuchten Modellorganismen hinaus, weitere detaillierte Untersuchungen an anderen Species. Im Rahmen dieser Arbeit beschreibe ich die Etablierung der Keimbahn bei dem Polychaeten *Platynereis dumerilii*, einem aufstrebendem Modellorganismus, der gut für vergleichende entwicklungsbiologische Studien innerhalb der Bilateria geeignet ist.

Im ersten Teil beschreibe ich die Entwicklung der Keimbahn von *Platynereis* mit Hilfe von verschiedenen molekularen Keimzellmarkern, einschließlich *vasa*, *nanos* und *par-1*.

Diese Analyse ergab, dass die primordialen Keimzellen mesodermalen Ursprungs sind und zum Ende der Larvalentwicklung hin aus einer Region undifferenzierter Zellen auswandern; der so genannten posterioren Wachstumszone. In Jungtieren ruhen die primordialen Keimzellen zunächst in den ehemaligen Larvalsegmenten, während der Polychaet durch Hinzufügen neuer Segmente von der posterioren Wachstumszone her weiter wächst. Mit Eintritt der Gametogenese beginnen diese Zellen zu proliferieren, wandern zu den hinteren Segmenten und differenzieren sich dort zu Gameten.

Im zweiten Abschnitt weitete ich die Expressionsanalyse von Keimbahn-Genen auf zwei noch nicht endgültig differenzierte Gewebetypen aus: der Wachstumszone und regenerierenden Hinterenden. Basierend auf dem Expressionsmuster von *vasa* in diesen Geweben im Vergleich zu verschiedenen Genen, die bekanntermaßen in totipotenten- und Stammzellen eine Rolle spielen, wie *pl10* und *piwi*, schließe ich, dass eine ursprüngliche Verbindung zwischen diesen Prozessen besteht, die bei *Platynereis* erhalten ist.

Der dritte Teil meiner Arbeit beschäftigt sich mit dem Zusammenhang zwischen Keimbahndetermination und Achsenbildung. Hier habe ich die Expressionsmuster von *nanos* – einem der posterioren Gene bei *Drosophila*, welches sowohl für die Aufrechterhaltung des Keimzellschicksals als auch für die Entwicklung der posterioren Segmente verantwortlich ist – mit der mRNA Verteilung des *Gap*-Gens *hunchback* in der frühen Larve verglichen. Meine Untersuchungen bestätigen eine mögliche konservierte Rolle von Nanos (wahrscheinlich zusammen mit seinem Partner Pumilio) bei der Hemmung der Translation von *hunchback*, wie sie auch bei *Drosophila* zu finden ist. Dies legt nahe, dass dieses System einen ursprünglichen Mechanismus zur Etablierung der Polarität in Embryos von Protostomiern darstellt.

Erklärung

Ich versichere, daβ ich meine Dissertation

"Germline development in *Platynereis dumerilii* and its connection to embryonic patterning"

selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Heidelberg, den 3. März 2004

Yanire Fabiola Zelada González